University of Alberta

Maturation of Fatty Acid Oxidation in the Newborn Heart

By

Arzu Onay-Besikci ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial
fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Pharmacology

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18 July 2003

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Maturation of Fatty Acid Oxidation in the Newborn Heart* submitted by *Arzu Onay-Besikci* in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

18 July 2003

Dr. Philipp Scherer

Dedication

My mother, Semsinur Onay

She taught me to keep my eyes on the sky and my feet on the ground
Mom, I wish I'll become just like you when I grow up

Abstract

Energy in the form of ATP is supplied from the oxidation of fatty acids and glucose in the adult heart. In the fetal heart, lactate oxidation and glycolysis are preferred source for ATP production. As the newborn heart matures, fatty acid oxidation increases and becomes the dominant oxidative substrate for the heart. This thesis examined the role of malonyl CoA inhibition of carnitine palmitoyltransferase I (CPT I) in this process. Malonyl CoA levels decrease following birth, resulting in a decrease in malonyl CoA inhibition of CPT I. Using 7-day-old rabbit hearts, we demonstrate that a decrease in malonyl CoA, as opposed to changes in L-carnitine content, is responsible for the increase in fatty acid oxidation in the newborn heart. To emphasize the regulatory role of malonyl CoA, fatty acid oxidation was stimulated by increasing fatty acid supply to the myocardium. Even with fatty acid oxidation being a major source of energy, increasing fatty acid concentration further decreased malonyl CoA levels and increased fatty acid oxidation. Adiponectin is a peptide hormone that is secreted by differentiated adipocytes. A proteolytic cleavage product of adiponectin, gAd, increases fatty acid oxidation in skeletal muscle and cardiac myocytes by stimulating AMP-activated protein kinase (AMPK). AMPK phosphorylates and inactivates acetyl CoA carboxylase (ACC), the enzyme that is involved in malonyl CoA synthesis. We examined if adiponectin or gAd play a role in the maturation of fatty acid oxidation in the newborn rabbit heart. Plasma adiponectin increased in newborn rabbits following birth. Since plasma insulin levels are low throughout the suckling period, we examined the effects of adiponectin and gAd on fatty acid oxidation in hearts perfused in the presence or absence of insulin. Adiponectin and gAd did not alter fatty acid oxidation in the presence of insulin. However, in the absence of insulin, gAd increased fatty acid oxidation compared to control. This increase in fatty acid oxidation occurred independent of changes in AMPK, ACC and malonyl CoA. These results suggest that a decrease in plasma insulin and increase in gAd are involved in the increase of cardiac fatty acid oxidation in the immediate newborn period.

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List of Abbreviations

ACBP	Acyl CoA binding protein
ACC	Acetyl CoA carboxylase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AMPKK	AMPK-kinase
AT	Angiotensin
ATP	Adenosine triphosphate
CAT	Carnitine acylcarnitine translocase
CoA	Coenzyme A
CoASH	Free Coenzyme A
CPT I	Carnitine palmitoyltransferase I
CPT II	Carnitine palmitoyltransferase II
DCA	dichloroacetate
EDL	Extensor digitorum longus
FABP	Fatty acid binding protein
FABP_{pm}	Plasma membrane fatty acid binding protein
FAD^+	Flavin adenine dinucleotide
FADH	Reduced form of FAD^+
FAT	Fatty acid translocase
FPLC	Fast performance liquid chromatography
gAd	Globular head domain of adiponectin
GH	Growth hormone
g	Gram
IC50	Half-maximal inhibitory concentration
Km	Affinity constant
LCAS	Long chain acyl CoA synthase

L-CPT I	Liver isoform of CPT I
LDH	Lactate dehydrogenase
MCD	Malonyl CoA decarboxylase
M-CPT I	Muscle isoform of CPT I
mg	Milligram
min	Minute
ml	Milliliter
NAD^+	Nicotinamide adenine dinucleotide
$NADH+H^+$	Reduced form of NAD^+
n	Nano (10^{-9})
PDC	Pyruvate decarboxylase
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PDP	Pyruvate dehydrogenase phosphatase
PFK	Phosphofructokinase
PKA	Protein kinase A
PPAR	Peroxysome proliferator-activated receptor
TNF	Tumor necrosis factor
V	Enzyme velocity
VLACS	Very long chain acyl CoA synthase
Vmax	Maximal enzyme velocity
μ	Micro (10^{-6})

Chapter 1

Introduction

1.1. Introduction

The heart has a very high demand for energy. Energy in the form of ATP is primarily supplied from the oxidation of fatty acids and glucose in the adult heart. In the fetal heart on the other hand, lactate oxidation and glycolysis are preferred sources for ATP production (Lopaschuk and Spafford 1990, Lopaschuk et al 1991, Werner and Sicard 1987). As the newborn heart matures, fatty acid oxidation increases and becomes the dominant oxidative substrate for the heart in most mammalian species.

The adaptation of the newborn heart to the extrauterine life is a fascinating and complex process. The increasing work requirement and high growth rate of the heart creates the necessity for increased energy substrates. The high fatty acid content of the maternal milk in many species is an effective way to supply the high energy demand of the newborn heart.

The mechanism for this switch in energy substrate preference is a combination of changes of postnatal nutrition and environment, as well as direct subcellular changes within the myocardium. An increase in mitochondrial uptake of fatty acids has been implicated in this process (Girard 1992, Lopaschuk et al 1994, Brown et al 1995). The transport of fatty acids into the mitochondria, is primarily controlled at the level of carnitine palmitoyltransferase I (CPT I) (McGarry and Foster 1980). CPT I is in turn inhibited by malonyl CoA and stimulated by L-carnitine (McGarry and Foster 1980, McGarry et al 1983, Weis et al 1994, Brown et al 1995). The heart expresses two isoforms of CPT I, L-CPT I and M-CPT I, with L-CPT I being less sensitive to inhibition by malonyl CoA, and requiring lower L-carnitine concentrations for maximal stimulation (McGarry and Foster, 1980, McGarry et al 1983, Weis et al 1994).

The dramatic increase in fatty acid oxidation seen in the newborn heart has been attributed to a postnatal increase in L-carnitine levels and a switch from the L-CPT I isoform to the M-CPT I isoform (Brown et al 1995). However, since M-CPT I is more sensitive to inhibition by malonyl CoA this switch should actually lead to a decrease in fatty acid oxidation rates, not an increase as previously observed.

An alternate explanation for the increase in fatty acid oxidation is a decrease in myocardial malonyl CoA levels following birth, resulting in a decrease in malonyl CoA inhibition of CPT I. Indeed, our laboratory has previously shown that following birth, malonyl CoA levels dramatically decrease in the newborn heart. In one-day old rabbit hearts, malonyl CoA levels are very high, but decrease dramatically by 7-days following birth (Lopaschuk et al 1994a, Lopaschuk et al 1994b, Dyck et al 1998, Makinde et al 1997). These changes in malonyl CoA can be explained by alterations in the control of both malonyl CoA synthesis and degradation (Lopaschuk et al 1994a, Lopaschuk et al 1994b, Dyck et al 1998, Makinde et al 1997).

The hormonal environment to which the newborn heart is exposed also changes in the newborn period. During fetal life, circulating levels of insulin are high and glucagon levels are quite low. Insulin levels decrease and glucagon levels rise immediately after birth. The alterations in the levels of the energy substrates and enzyme activities coupled with the changes in hormonal levels contribute to the adaptation of the neonate to the life *ex utero*.

Adiponectin is an adipocyte-derived hormone that has recently been shown to play a role in insulin function and energy homeostasis (Scherer et al 1995). Adiponectin facilitates the effect of insulin on hepatic glucose production *in vitro* (Berg et al 2001). *In vivo*, administration of adiponectin reduces basal plasma glucose levels (Combs et al 2001, Yamauchi et al 2001). A proteolytic cleavage product of adiponectin, gAd, has also been shown to have physiological effects. Both gAd and full-length adiponectin increase fatty acid oxidation in muscle and cause weight loss in mice (Fruebis et al 2001). Recently, Yamauchi et al (2002) demonstrated that gAd and full-length adiponectin stimulates 5'-AMP-activated protein kinase (AMPK) in skeletal muscle. The authors showed that this activation increased the phosphorylation of acetyl CoA carboxylase (ACC), *increased fatty acid oxidation*, and increased glucose uptake and lactate production in C2C12 myocytes. When administrated *in vivo*, both full-length adiponectin and gAd stimulate AMPK and increase the phosphorylation of ACC in mouse soleus muscle (Yamauchi et al 2002). Moreover, stimulation of AMPK with full-length adiponectin reduces gluconeogenesis in the liver and plasma glucose levels (Yamauchi et al 2002). In agreement with these findings, Tomas et al (2002) reported that gAd stimulates fatty acid oxidation in skeletal muscle by activating AMPK and inactivating ACC.

We have shown that the plasma levels of adiponectin are very low in 1-day old rabbits and increase to 10-15 μg/ml in 7-day old rabbits. The effect of adiponectin on the regulation of fatty acid and glucose metabolism in the newborn heart is a part of the studies presented in this thesis (Chapter 5).

High levels of insulin possibly functions to set the metabolic pathways in favor of biosynthetic activities in the fetal period. Moreover, high insulin levels in fetal life may be expected, as the fetus needs to be able to efficiently transport glucose from the maternal circulation. A significant drop in insulin that is seen immediately after birth may trigger other hormonal and metabolic responses which the newborn requires for its successful adaptation to the extrauterine life.

In this chapter, we will first outline the pathways that are involved in energy production. We will summarize the subcellular regulation of fatty acid metabolism, and the regulatory role of malonyl CoA. We will briefly discuss the involvement of malonyl CoA in the regulation of fatty acid oxidation other than that proposed for the newborn period. We will also review the current knowledge in the maturation of fatty acid oxidation in the newborn heart, and the implications for myocardial ischemia. Finally, we will describe the actions of adiponectin that are involved in the intermediary metabolism.

1.2. Energy Production in the Heart

The heart has a very high energy demand due to the continuous work performed by the cardiac muscle. Under normal physiological conditions, the main fuels involved in maintaining the high energy requirements of the heart are fatty acids, glucose, and lactate (Figure 1.1). Glucose metabolism starts with the reactions of glycolysis in the cytoplasm. Under aerobic conditions, the pyruvate generated from glycolysis is taken up by the mitochondria and is converted to acetyl CoA by the pyruvate dehydrogenase complex (PDC). Lactate can also be taken up by the heart and converted to pyruvate via lactate dehydrogenase (LDH).

Although glucose is an important source for ATP production, under normal physiological conditions the adult heart obtains approximately 60-80% of its overall energy requirements from the oxidation of fatty acids (Shipp et al 1961, Neely and Morgan 1974). Fatty acids cross plasma membrane by diffusion or by protein-mediated transport (reviewed in Schaffer 2002 and Eaton 2002). Once in the cytoplasm, fatty acids are converted to their CoA esters, a process known as the activation of fatty acids. The acyl moiety is then transferred into the mitochondria by a complex of enzymes involving carnitine palmitoyltransferase I (CPT I), carnitine translocase, and carnitine palmitoyltransferase II (CPT II) (Figure 1.1). Once inside the mitochondrial matrix, acyl CoA passes through β-oxidation. Each successive cycle of the β-oxidation produces one molecule of acetyl CoA, and one of each NADH and $FADH_2$.

Acetyl CoA derived from glucose, fatty acid, or lactate then enters the Krebs' Cycle, resulting in the liberation of CO_2, NADH, and $FADH_2$. The NADH derived from glycolysis, the Krebs' Cycle, and β-oxidation, as well as $FADH_2$ from Krebs' Cycle and β-oxidation then enter the electron transfer chain. The H^+ on NADH and $FADH_2$ is transferred to H_2O in the presence of O_2 and ADP is converted to ATP (Figure 1.1).

1.3. Subcellular Regulation of Fatty Acid Oxidation

1.3.1. Fatty Acid Transport across Plasma Membrane

The importance of subcellular regulation of fatty acid oxidation is demonstrated by the delay in the increase of fatty acid oxidation despite a sharp increase in plasma fatty acids and glucagon and a fall in plasma insulin levels. Fatty acids cross the plasma membrane by diffusion or by protein-mediated transport (reviewed in Schaffer et al 2002 and Eaton et al 2002). Three main types of tissue-specific putative fatty acid binding and transport proteins have been identified: the plasma membrane fatty acid binding protein ($FABP_{pm}$) (Stremmel et al 1985); fatty acid translocase (FAT)/CD36 (Harmon et al 1991); and fatty acid transport proteins (Stahl et al 2001). Cytosolic fatty acid binding proteins (FABP) have a similar role to that of plasma albumin in the transfer of hydrophobic fatty acids within the cytosol. Another cytosolic protein, acyl CoA binding protein (ACBP), that has higher affinity for acyl CoA esters of 14-22 Carbon chain length and a lower affinity for fatty acids and carnitine esters has also been isolated (Rosendal et al 1993). High affinity for acyl CoA of ACBP is unlikely to be a limiting factor for mitochondrial β-oxidation since ACBP-bound acyl CoA has been shown to be a substrate for carnitine palmitoyltransferase I (CPT I) (Abo-Hashema et al 1999). The evidence for a role of ACBP *in vivo* requires further investigation.

1.3.2. Activation of fatty acids

Activation of long-chain fatty acids by esterification with CoA at the C-1 position is necessary for anabolic and catabolic processes in which fatty acids are utilized (Watkins 1997). Acyl CoA synthases vary in their affinities for fatty acids with different chain lengths and their tissue localization (Fujino et al 1996, Tang et al 2001). In a study by Coe et al (1999), co-localization of very long chain acyl CoA synthase (VLACS) activity with FATP in Cos7 cells suggests that FATP contained transport and esterification activity. Similarly, several members of the FATP family of proteins have been found to have acyl CoA synthase activity (Coleman et al 1992, Herrmann et al 2001, Uchiyama et al 1996). Although their substrate specificity still remains unclear, the wide range of fatty acyl CoA synthases suggests that there may be a channeling of acyl CoA esters towards β-oxidation or esterification. Support for this came from studies where LCAS and

CPT I were shown to be enriched in the contact sites of the mitochondrial inner and outer membranes (Hoppel et al 1997). Thus, contact sites were proposed to provide the necessary physical interaction for the enzymes that facilitate fatty acid transport into the mitochondria (reviewed in Kerner and Hoppel 2000).

Although activation of fatty acids is not considered to be a major site for regulation of fatty acid oxidation, this process may still play a role. Since the cytosolic concentration of free CoA (CoASH) is equal or less than the Km of the enzyme, the activity of the acyl CoA synthase has been suggested to be regulated by the availability of its substrate (CoASH) (Oram et al 1973). Direct comparison of the inhibition of acyl CoA synthase and β-oxidation *in vivo* is not conclusive due to the lack of a specific inhibitor of the enzyme.

1.3.3. Carnitine Palmitoyltransferase I and the Regulation of Fatty Acid Import into the Mitochondria

Control of fatty acid oxidation appears to be primarily exerted at the level of transport of fatty acids into the mitochondria. CPT I catalyzes the formation of long-chain fatty acylcarnitines from fatty acids and free carnitine. Malonyl CoA, the substrate for CPT I in hepatic lipogenesis, is also a potent physiological inhibitor of the enzyme (McGarry and Foster 1980). Evidence from studies in intact tissues and cells suggests a primary regulatory role of malonyl CoA in the regulation of fatty acid oxidation.

CPT I exists in two isoforms. The liver isoform (L-CPT I or CPT Iα) displays a higher affinity for carnitine and is less sensitive to the inhibition by malonyl CoA compared to the muscle isoform (M-CPT I or CPT Iß), whereas their affinities for long-chain acyl CoA are similar (Saggerson and Carpenter 1982, McGarry et al 1983). The isoforms also show different tissue distribution. L-CPT I is widely expressed with the highest level of expression in the liver, kidney, ovary, intestine, pancreatic islet cells, spleen, and in the heart. M-CPT I is the dominant isoform in skeletal muscle, brown and white adipose tissue, adult heart, and testis (McGarry and Brown 1997, Kerner and Hoppel 1998, Nicot et al 2001). Human fibroblasts express only L-CPT I (Britton et al 1995).

CPT I is an integral protein that has two transmembrane domains (Figure 1.2) (Fraser et al 1997). Both N-terminus and the catalytic C-terminus are exposed to the cytosol (Fraser et al 1996, Fraser et al 1997, Zammit et al 1997). Two transmembrane segments are connected with a short loop of 27 amino acids that is localized in the intermembrane space between the outer and inner mitochondrial membranes (Fraser 1997 et al). The extreme N-terminus of the protein is necessary for the malonyl CoA sensitivity. Malonyl CoA sensitivity of L-CPT I is completely lost by the deletion of 6 highly conserved amino acid residues (Shi et al 1998). Jackson et al (2000a) showed that a short segment within the N-terminus has a strong negative effect on malonyl CoA sensitivity. Deletion of this region increased malonyl CoA sensitivity by 50-fold (Jackson et al 2000a). In the same study, the authors also shed light on the unexplained feature of the L- and M-CPT I isoforms in their inverse relationship between the sensitivity to the inhibition by malonyl CoA and affinity for carnitine (McGarry et al 1983). They showed that the extreme N-terminus has reciprocal effects on affinities for carnitine and for malonyl CoA: this sequence, although entirely conserved between the two isofoms, controls IC_{50} for malonyl CoA in L-CPT I (but not M-CPT I), whereas it controls the Km for carnitine in M-CPT I but not in L-CPT I (Jackson et al 2000b). The interaction between the N- and C-termini is important for malonyl CoA sensitivity. The same group constructed chimeras using combinations of three segments (N-terminus plus first transmembrane domain, loop plus second transmembrane segment, and C-terminus) from each of L- and M-CPT 1 (Jackson et al 2000a). They showed that the precise N-terminus to C-terminus pairings affected the sensitivity to malonyl CoA and the Km for palmitoyl CoA of the chimeric CPTs, whereas the interaction between the transmembrane domains affected the affinity for carnitine (Jackson et al 2000a).

Interestingly, when expressed in *Pichia pastoris,* L-CPT I from pig showed a much higher sensitivity to malonyl CoA inhibition compared to human or rat L-CPT I. The affinity for carnitine and palmitoyl CoA of pig L-CPT I was similar to human and rat L-CPT (Nicot et al 2001). However, the physiological importance of this natural chimera of L-CPT I is unknown.

A novel gene that encodes a protein that belongs to the CPT I family has recently been shown (Price et al 2002). This new gene was named CPT IC since it is related to CPT IA and CPT IB, the genes encoding the liver and muscle isoforms of CPT I. *In situ* hybridization on mouse coronal brain sections showed a general low-level distribution of CPT IC mRNA. However, extremely high levels of mRNA were concentrated in discrete areas that have been implicated in appetite control or feeding behavior. When expressed in yeast, this protein did not show catalytic activity with the known substrates of L-and M-CPT I, however it displayed high affinity for malonyl CoA at physiological concentrations. Interestingly, a role for malonyl CoA has been suggested recently in appetite control (Loftus et al 2000). Recently, Wortman et al (2003) challenged the role of increased malonyl CoA and subsequent build up of long-chain fatty acids in the anorexic effect of a synthetic fatty acid synthase inhibitor C75. They tested the hypothesis that increased glucose use, rather than decreased lipid use *per se*, is necessary for the anorexic effects of C75. They proposed that C75 does not inhibit food intake in situations where the provision of alternative fuels (increased ketone body usage, or icv injection of lactate or glutamine) mitigates increased demand for glucose. Together with the tissue distribution, this finding introduces an exciting route for malonyl CoA-regulated appetite control. CPT IC may be a unique protein that the brain requires for highly specialized lipid metabolism. The substrates and products as well as the physiological function of this protein require further investigation.

There are two binding sites present in both L- and M-CPT I (Shi et al 1999, Shi et al 2000). The low-affinity binding site is near the catalytic acyl CoA binding domain. Malonyl CoA as well as other compounds with CoA moieties, such as acetyl CoA, glutaryl CoA, or free CoA could exert inhibitory effect by binding to this site (Zierz and Engel 1987). The high-affinity binding site does not compete with acyl CoA and behaves as an allosteric component (Bird and Saggerson 1984, Cook et al 1994, Kashfi et al 1994). Although present within the general outer membrane of mitochondria, CPT I has been proposed to be concentrated within the contact sites between the outer and inner membranes (Fraser and Zammit 1998). Mitochondrial sub-fractionation studies suggested that the kinetic characteristics are also dramatically different between the CPT I localized to the contact sites and to the outer membrane enzyme. In the outer membrane, malonyl CoA inhibition affects V and not the Km for palmitoyl CoA, whereas in the contact sites V is unchanged but the Km for palmitoyl CoA is increased (Fraser et al 2001). Consequently, inhibition by malonyl CoA in the contact sites appears to be competitive (Fraser et al 2001). Contact sites are defined areas of outer and inner membranes that are in a very stable contact and cannot be separated by mechanical forces (Brdiczka et al 1986). At these sites, mitochondrial inner and outer membranes come to within 4 nm of each other, and they make up for the 5-10% of the outer membrane (Pfanner 1992). The presence of about 40% of both CPT I and CPT II has been suggested to play a role in the transport of acylcarnitines to the mitochondria (Brdczka 1991, Fraser and Zammit 1998). Similarly, Hoppel's group prepared mitoplasts with an intact inner mitochondrial membrane and CPT I activity by French press treatment (Hoppel et al 1998). Morphological examination of these mitoplasts showed that the outer membrane retained in these preparations contained the contact sites. They observed that long-chain acyl CoA synthase (LCAS) and CPT I are disproportionally retained when compared to the other membrane markers (Hoppel et al 1998). Competitive inhibition of CPT I by malonyl CoA in these contact sites would mean that an increase in acyl CoA could overcome this inhibition and prevent the accumulation. In the outer membrane, non-competitive inhibition would ensure that acyl CoA that is required for other cytosolic functions is not converted to acylcarnitine (Ramsay et al 2001).

The finding that a decrease in pH which is associated with the protonation of the imidazole group of histidine indicated the involvement of this amino acid in the interaction with malonyl CoA (Stephens et al 1983, Mills et al 1984, Morillas et al 2000). In a recent study, His 483 on CPT I has been shown to be responsible for malonyl CoA sensitivity of L-CPT I (Morillas et al 2002). Alanine (Ala478 in CPT I) is the first amino acid after the acidic residue and is conserved in all malonyl CoA-inhibitable carnitine acyltransferases but is absent in non-malonyl CoA-inhibitable carnitine acyltransferases. In the latter group of enzymes, this position is substituted with glycine (Morillas et al 2002). When Ala478 was mutated to glycine and expressed in S. cerevisia (an organism devoid of acyl CoA transferase activity), IC_{50} of the mutant was increased compared to the wild type. These results indicate the involvement of Ala478 in malonyl CoA sensitivity of L-CPT I (Morillas et al 2002).

Examination of the primary structures of the carnitine acyltransferases revealed another completely conserved histidine residue as the candidate for the catalytic site (Brown et al 1994). According to the recent theory by McGarry's group, the hydroxyl group of the carnitine is deprotonated by this histidine residue. An aspartate residue that is conserved amongst the carnitine acyltransferases acts as a charge relay in this system (Brown et al 1994).

1.4. Regulation of Malonyl CoA Levels in the Heart

1.4.1. Malonyl CoA synthesis

Malonyl CoA in the heart is produced by the carboxylation of acetyl CoA by acetyl CoA carboxylase (ACC). Two major isoforms of ACC have been identified, a 265-kDa (ACC265, ACC1 or ACCα) and a 280-kDa (ACC280, ACC2 or ACCβ) isoform. Both isoforms are expressed in the heart (Abu-Elheiga et al 1997, Lopaschuk et al 1994b). The two isoforms are distinct gene products

(Abu-Elheiga et al 1995, Abu-Elheiga et al 1997). The major difference between the isoforms is the extended N-terminus of ACC280. In lipogenic tissues, such as white adipose tissue and lactating mammary gland, ACC265 is the dominant form expressed. In oxidative tissues, such as rat heart and skeletal muscle, ACC280 predominates. Having said that, an approximate equal distribution of both isoforms is observed in the rabbit heart (Lopaschuk et al 1994b). The significance of this differential species distribution between ACC265 and ACC280 is not known. It is also unknown if these two isoforms have different roles in the heart. Although the 280 isoform of both skeletal muscle and heart ACC have been grouped together and called ACC280, it is not entirely clear whether heart and skeletal muscle ACC are identical.

Studies from our laboratory in isolated working hearts show that ACC is directly involved in the regulation of fatty acid oxidation (Saddik et al 1993, Kudo et al 1995, Kudo et al 1996, Lopaschuk et al 1994b, Makinde et al 1997). The role of ACC in the regulation of skeletal muscle fatty acid oxidation has also been shown (Merrill et al 1997, Saha et al 2000). The importance of the regulation of ACC in muscle was recently shown in a report indicating that knockout mice lacking the muscle isoform of ACC have less fat accumulation in adipose tissue and higher rates of fatty acid oxidation in skeletal muscle compared to normal mice (Abu-Elheiga et al 2001). These high fatty acid oxidation rates correlate with a decrease in malonyl CoA levels, which highlights the role of ACC280-produced malonyl CoA in the regulation of fatty acid oxidation.

One of the key mechanisms by which cardiac ACC is regulated is via phosphorylation and inhibition by AMP-activated protein kinase (AMPK) and by protein kinase A (PKA). AMPK is a serine/threonine kinase that responds to metabolic stresses that deplete cellular ATP (reviewed in Hardie and Carling 1997). Under conditions of metabolic stress, AMPK is activated and inhibits energy requiring anabolic processes such as lipid liposynthesis. AMPK is a heterotrimetic enzyme consisting of α, β, and γ subunits (Hardie and Carling

1997). Each subunit has more than one isoform. So far, two isoforms for α (α_1 and α_2), two isoforms for β (β_1 and β_2), and three isoforms for γ (γ_1, γ_2, and γ_3) subunits have been identified (Beri et al 1994, Hardie et al 1998, Cheung et al 2000). The heart expresses all of these isoforms except the α3 isoform that is localized to skeletal muscle. These isoforms appear to play a role in a tissue-specific manner. The α-subunit is the catalytic subunit containing the kinase domain that transfers a phosphate from ATP to the target protein (Hardie and Carling 1997). The β- and γ-subunits are considered regulatory components of the enzyme (Hardie and Carling 1997, Cheung et al 2000).

Our laboratory characterized AMPK activity in the heart tissue and found that AMPK activity is comparable to that in the liver (Kudo et al 1995, Kudo et al 1996). AMPK itself is regulated by both allosteric and covalent mechanisms. An increase in AMP results in the allosteric activation of AMPK. AMP binding makes AMPK a better substrate for the phosphorylation by an upstream kinase, AMPK-kinase (AMPKK) (Hawley et al 1995, Hamilton et al 2002). AMP also directly activates AMPKK (Hawley et al 1996). Initially, this activation has been shown to phosphorylate AMPK at Thr172 on the α-subunit (Hawley et al 1996). However, recent evidence suggests that AMPKK phosphorylates both α- and β-subunits of AMPK (Warden et al 2001). In addition to making AMPK a better substrate for AMPKK, AMP binding also makes AMPK a poor substrate for protein phosphatase 2C, the phosphatase that appears to be responsible for dephosphorylation of AMPK and thus keeps AMPK in the active state (Davies et al 1995). Recently, AMPK activation independent of AMP:ATP ratio has been demonstrated (Beauloye et al 2001, Daniel and Carling 2002).

Once activated, AMPK phosphorylates a number of targets resulting in increases in glucose transport and fatty acid oxidation (Hardie and Carling 1997, Ruderman et al 1999, Mu et al 2001). Of importance to fatty acid oxidation in the heart is that AMPK can phosphorylate and inactivate ACC (Witters and Kemp 1992, Witters et al 1994, Park et al 2002). Both AMPK and PKA can phosphorylate ACC *in vitro*. In rat ACC265, AMPK phosphorylates serine residues 79, 1200, and 1215 and PKA phosphorylates serine residues 77 and 1200 (Munday et al 1988). Hardie's group has shown that the removal of the N-terminus of ACC265 phophorylated by either kinase results in the full activation of ACC (Davies et al 1990). Based on this finding, they suggested that only Ser77 and Ser79 have an inhibitory effect on ACC activity upon phosphorylation. However, Ha and co-workers have mutated Ser79 and Ser1200 and shown that only the latter mutation abolished the inhibitory effects of PKA (Ha et al 1994). The relative importance of phosphorylation of Ser79 and Ser1200 to the regulation of ACC activity remains an open question.

PKA phosphorylation of ACC280 *in vitro* has been shown to produce significant inactivation (Dyck et al 1999) or have no effect on activity (Boone et al 1999). However, it is clear that ACC280 can also be phosphorylated by PKA. Whether these conflicting results between studies are due to two possible distinct isoforms in skeletal muscle and the heart is not known.

Citrate is an allosteric activator of ACC *in vivo* (Munday et al 1988). Increases in malonyl CoA concentration are not always accompanied by measurable changes in ACC activity (Saha et al 1999, Bavenholm et al 2000). However, there is a strong positive correlation between malonyl CoA concentration and increased concentrations of citrate and malate. Therefore, allosteric activation of ACC280 by citrate rather than a change in its phosphorylation state is proposed to be responsible in the increase in malonyl CoA levels (Saha et al 1999, Bavenholm et al 2000).

1.4.2. Malonyl CoA degradation

Unlike lipogenic tissues where malonyl CoA can be metabolized by fatty acid synthase or fatty acid elongases, the heart does not contain measurable activities of these enzymes (Alam and Saggerson, 1998). In the heart, our laboratory has proposed that the main pathway for removal of malonyl CoA is via degradation by malonyl CoA decarboxylase (MCD), which decarboxlyates malonyl CoA to acetyl CoA (Figure 1.2). Studies from our laboratory showed that the heart has an active MCD (Dyck et al 1998). Although originally reported to be a mitochondrial enzyme (Kolattukudy et al 1981), recent evidence suggests that MCD has multiple intracellular locations such as peroxisomes, mitochondria and cytosol (Alam and Saggerson 1998, Sacksteder et al 1999, Dyck et al 2000). Determination of the subcellular localization of this enzyme will increase our understanding as to how β-oxidation continues despite a high intracellular malonyl CoA concentration.

Our laboratory has purified MCD and showed that heart MCD is expressed as a 50 kDa protein that forms a tetramer in the intact cell (Dyck et al 2000). In collaboration with Dr. Marc Prentki (Univeristy of Montreal), our laboratory cloned and sequenced rat MCD (Voilley et al, 1999, Dyck et al 2000). The human MCD cDNA has also now been sequenced (Gao et al 1999). MCD has two putative 5' start sites that encode a 54 or a 50 kDa protein. While islet cells express both isoforms of MCD, cardiac cells primarily express the 50 kDa isoform (Dyck et al 2000). Sequence analysis of MCD suggests that a mitochondrial targeting sequence exists on the amino terminus. Both isoforms of MCD are expressed in cardiac cells, with the 50 kDa isoform localized to the mitochondria (Sambandam et al, manuscript submitted).

Since the initial identification of MCD in cardiac muscle, a number of studies have implicated MCD as being an important regulator of fatty acid oxidation. In the heart, conditions in which fatty acid oxidation increases are associated with high MCD activity, including fasting, diabetes, ischemia, increased work, and newborn heart development (Dyck et al 1998, Goodwin and Taegtmeyer 1999, Dyck et al 2000, Sakamoto et al 2000). In skeletal muscle, liver, and pancreatic islet cells, high MCD activity is also associated with high fatty acid oxidation rates (Antinozzi et al 1998, Park et al 2002, Ruderman and Dean 1998, Saha et al 2000).

MCD is subjected to both acute regulation (i.e. phosphorylation control by as yet unidentified kinases) (Dyck et al 1998) and by chronic transcriptional control (Campbell et al 2002). The expression and activity of cardiac MCD is increased in diabetes, fasting, high fat feeding and newborn hearts (Awan et al 2000, Dyck et al 1998, Goodwin and Taegtmeyer 1999, Dyck et al 2000, Sakamoto et al 2000). Part of this transcriptional control is regulated by PPARα (Campbell et al 2002). In PPARα null mice, MCD expression and activity is decreased (Campbell et al 2002). This is accompanied by a decrease in fatty acid oxidation rates.

1.5. Pathologies That Modify Malonyl CoA Levels in The Heart

It is increasingly becoming recognized that alterations in fatty acid oxidation contribute to a number of cardiac pathologies, including ischemic heart disease, diabetic cardiomyopathies, cardiac hypertrophy and heart failure. Table 1.1 summarizes these conditions and treatments that modify malonyl CoA levels and fatty acid oxidation rates in the hearts.

1.5.1. Ischemia-induced Changes in Malonyl CoA Control of Fatty Acid Oxidation

During ischemia, AMPK is rapidly phosphorylated and activated (Kudo et al 1995, Kudo et al 1996, Beauloye et al 2001). AMPK is also activated during both mild and severe ischemia (J.Y. Altarejos and G.D. Lopaschuk, unpublished observations). This is accompanied by an increase in T172 phosphorylation of the α catalytic subunit of AMPK, and an increase in AMPK activity. This activation of AMPK is due in part to an ischemia-induced increase in the ratio of AMP/ATP and Cr/PCr. However, AMPK activation can occur independent of chages in nucleotide levels (Beauloye et al 2001).

Activation of AMPK during ischemia may represent an attempt by the myocyte to increase its energy supply. This includes a stimulation of fatty acid oxidation (Kudo et al 1995, Kudo et al 1996). In addition, AMPK also promotes glucose uptake and glycolysis during ischemia (Russell et al 1998, Russell et al

1999, Beauloye et al 2001). AMPK activation promotes the translocation of GLUT-4 to the sarcolemma of the myocyte (Russell et al 1998, Russell et al 1999) and also phosphorylates and activates phosphofructokinase kinase 2 (Beauloye et al 2001, Marsin et al 2000), which produces fructose 2,6-bisphosphate, a potent stimulator of glycolysis. As a result, AMPK may be an important mediator of the increased glycolysis that occurs during ischemia. However, one of the consequences of this is an accelerated production of deleterious glycolytic by-products, particularly lactate and protons. Although overall oxidative metabolism during ischemia is primarily dependent on the degree of ischemia (i.e. oxygen supply), activation of AMPK during ischemia could result in fatty acid oxidation dominating, at the expense of glucose oxidation, as the source of residual oxidative metabolism (Lopaschuk 2002).

Upon reperfusion of the reversibly damaged ischemic myocardium, AMPK activation persists (Kudo et al 1995, Kudo et al 1996). The reperfused heart is also exposed to high levels of fatty acids in the plasma. This activation of AMPK and high circulating fatty acid levels have two consequences: 1) fatty acid oxidation recovers and provides the majority of the acetyl CoA for the Krebs' Cycle at the expense of glucose oxidation (Liedtke et al 1988, Lopaschuk et al 1990, Saddik and Lopaschuk 1991, Benzi and Lerch 1992) and 2) glycolytic rates remain elevated (Liu et al 1996, Liu et al 2002). As a result, proton production from uncoupled glucose metabolism persists into reperfusion, decreasing rates of pH recovery (Liu et al 2002).

During reperfusion of ischemic hearts, high AMPK activity correlates with a decrease in ACC activity, which is accompanied by a dramatic drop in malonyl CoA levels (Saddik et al 1993, Kudo et al 1995). We believe that this drop in malonyl CoA is primarily responsible for the high rates of fatty acid oxidation that occur during this period. However, of equal importance is that MCD activity is maintained (Awan and Saggerson 1993) during early reperfusion. A maintained degradation of malonyl CoA in the presence of decrease in malonyl CoA synthesis by ACC contributes to the dramatic decrease in malonyl CoA levels (Awan and Saggerson 1993). This drop in malonyl CoA concentration relieves the inhibition on CPT I and due to the high levels of circulating plasma fatty acid levels, fatty acids are almost unrestricted in their import into the mitochondria. This sets in motion the sequelae of events mentioned above that leads to enhanced ischemic damage.

1.5.2. Diabetes-induced Changes in Malonyl CoA Control of Fatty Acid Oxidation

In uncontrolled diabetics, myocardial glucose use is reduced and fatty acid oxidation accounts for most of myocardial oxygen consumption (Goodale et al 1959, Randle et al 1963, Wall and Lopaschuk 1989, Saddik and Lopaschuk 1991). This is due in part to a decrease in the number and transport of GLUT-4 to the sarcolemmal membrane. However, a major reason for the decrease in glucose metabolism is also the elevated levels of plasma fatty acids seen in the diabetic (Reaven et al 1988). Use of fatty acids for mitochondrial oxidative metabolism decreases both PDC activity and phosphofructose-1 kinase, key enzymes in glucose oxidation and glycolysis, respectively (Randle et al 1963, Neely and Morgan 1974, French et al 1988).

While high circulating levels of fatty acids in the diabetic decrease glucose metabolism, it is clear that other metabolic changes in the heart are also responsible for low rates of glucose metabolism. For instance, the decrease in PDC activity seen in diabetic rat hearts exceeds what would be expected if the inhibition occurred solely to a high level of circulating fatty acids (Randle et al 1963). This is supported by the observation that glucose oxidation rates are significantly lower in diabetic rat hearts compared to control hearts, even if hearts are perfused with similar concentrations of fatty acids (Randle et al 1963, Wall and Lopaschuk 1989). As a result, the combination of high levels of circulating fatty acids and direct alterations in insulin control of fatty acid and glucose oxidation can result in the diabetic rat heart becoming almost entirely dependent on fatty acid oxidation for its energy requirements (Goodale et al 1959, Randle et al 1963, Garland and Randle 1964, Wall and Lopaschuk 1989, Lopaschuk and Spafford 1991, Sakamoto et al 2000).

High circulating fatty acid levels and increased rates of cardiac fatty acid oxidation have been observed in a number of experimental models of diabetes. Our laboratory has shown that fatty acid oxidation rates are high in streptozotocin-diabetic rats (Sakamoto et al 2000), BB Wistar rats (Broderick et al 1992) and insulin-resistant JCR/LA rats (Lopaschuk and Russell 1991). Recent studies have also shown an increase in fatty acid oxidation contribution to energy production in db/db mice (Aasum et al 2003). This increase in fatty acid oxidation inhibits glucose oxidation and glycolysis (Aasum et al 2003) and contributes to a decrease in cardiac efficiency in the diabetic heart (Aasum et al 2003).

Under conditions of increased fatty acid oxidation such as diabetes and starvation, L-CPT I in the liver becomes more active and less sensitive to the inhibition by malonyl CoA. The decrease in the sensitivity is accompanied by a decrease in tissue of malonyl CoA that allows maximal fatty acid oxidation (Drynan et al 1996). The change in the sensitivity of CPT I to malonyl CoA is unique to the liver, but not to the muscle isoform of the enzyme (Mynatt et al 1992). The mechanism of this desensitization is not known. However, changes in the lipid environment of the membrane have been implicated by several studies (Zammit et al 1998). Desensitization of the enzyme also occurs when mitochondria are allowed to warm from 0°C to 22°C (McGarry and Brown 1997, Zammit et al 1984). Again, loss of sensitivity on warming has not been observed in adult rat heart (≈98% M-CPT I) (Weis et al 1994). If and how these *in vitro* changes in the sensitivity of CPT I relate to the decrease in sensitivity *in vivo* in diabetes and starvation is unknown.

MCD expression and activity in hearts from 6-week streptozotocin-induced diabetic rats are also significantly higher than control hearts (Sakamoto et al 2000). This suggests that an increased MCD activity contributes to the high fatty acid oxidation rates seen in diabetic hearts. This increase in MCD expression may involve an increase in PPARα activity in the diabetic (Leone et al 1998). PPARα is an important transcriptional regulator of MCD in the heart (Campbell et al 2002). In addition, overexpression of PPARα results in an increase in fatty acid oxidation in the mouse heart, and a phenotype similar to that seen in the diabetic mouse heart (Finck et al 2002b).

1.5.3. Control of Mitochondrial Fatty Acid Uptake in Cardiac Hypertrophy

Cardiac hypertrophy is another condition inwhich chronic alterations in malonyl CoA control of CPT I could contribute to alterations in energy metabolism. Previous studies have shown that the expression of genes encoding fatty acid oxidation enzymes is downregulated in the hypertrophied heart (Sack et al 1996, Sack et al 1997). This is accompanied with a shift of energy metabolism towards the fetal pattern (increase in ATP production from carbohydrates) (Sack et al 1996, Sack et al 1997, Depre et al 1998). Studies showed that many enzymes involved in the transport and oxidation of fatty acids such as fatty acid transport protein (FATP), fatty acid translocase (FAT), fatty acyl CoA synthase (FAS), CPT I, enoyl-CoA hydratase, and 3-hydroxy-acyl-CoA dehydrogenase are regulated by the expression of PPARα. Recently, PPARα regulation of MCD has been demonstrated by Young et al (2001). In that study, feeding rats with a PPARα ligand increased cardiac MCD expression. Further evidence that PPARα is involved in the regulation of MCD expression is provided by a study from our laboratory. Expression of both mRNA and protein as well as the activity of MCD significantly decreases in hearts from PPARα null mice. This was accompanied by an increase in the cardiac levels of malonyl CoA. The decrease in palmitate oxidation rate is compensated for by an increase in the rates of glucose oxidation and glycolysis (Campbell et al 2002).

1.6. Metabolic Changes in the Newborn Heart

Carbohydrates, especially lactate and glucose, are important energy substrates in the fetal heart. In the fetal heart, lactate oxidation and glycolysis are the preferred sources for ATP production (reviewed in Lopaschuk et al 1992, Makinde et al 1998). However, following birth fatty acid oxidation increases and becomes the predominant source of ATP production for the heart in most species. For example, in 1-day-old rabbits fatty acid oxidation provides only a small portion of the hearts energy requirements. However, in 14-day-old rabbits, fatty acids are the predominant source of energy. Further studies from our laboratory demonstrated that the increase in the ability of hearts to oxidize fatty acids occurs within the first week of life in rabbits (Lopaschuk et al 1990, Lopaschuk et al 1991).

The mechanisms responsible for this switch in energy substrate preference are due to a combination of events, including changes in energy substrate supply to the heart, changes in hormonal control of energy metabolism in the heart, and direct subcellular changes within the myocardium.

1.6.1. Energy Substrate Supply to the Fetal and Neonatal Heart

The newborn heart is confronted with the need to increase cardiac output to both the pulmonary and systemic circulation and operate against an increased vascular resistance. The increasing workload as well as the growth requirement of the newborn heart creates the necessity for increased energy substrate metabolism.

In utero, the fetus receives a constant supply of blood rich in carbohydrates (especially glucose and lactate) and amino acids and poor in fat (Battagglia and Meschia 1978, Girard et al 1992). Table 1.2 shows the amounts of the essential sources of energy and the levels of major hormones in the fetus, newborn and adult experimental animals. The placenta is a rich source for lactate during gestation (Comline and Silver 1976). Plasma levels of lactate exceed 10 mM in the fetus compared to the 1-2 mM in the newborn and even less in the adult

(Girard et al 1992). The placenta of most species is impermeable to free fatty acids. Accordingly, in most mammals the body fat content at birth is very low. In species in which free fatty acids are transferred across placenta (humans, guinea pigs, and rabbits), fatty acids in the form of triacylglycerol are distributed between liver and brown or white adipose tissue rather than oxidized by fetal tissues (Girard et al 1985). Plasma free fatty acid concentrations of the newborn reaches adult levels as soon as 2-3 hours after birth. This is due to the mobilization of the endogenous fat stores. Despite this abrupt increase in plasma free fatty acids, the increase in myocardial fatty acid oxidation appears after a lag period of 3-4 days in most species. This indicates that factors other than substrate supply also contribute to the initial increase in fatty acid oxidation in the newborn heart.

Several studies have shown that fetal hearts have a lower mitochondrial content and lower activity of Krebs' Cycle (Goodwin et al 1976, Hallman 1971, Werner et al 1982). This had led to the conclusion that the fetal heart has a lower oxidative capacity. Furthermore, low oxygen availability of heart in fetal life compared to life after birth has been suggested to contribute to a low oxidative capacity in the fetal heart. This may increase the contribution of glycolysis to total energy production in the fetal heart. However, Fisher et al (1981) showed myocardial oxygen consumption in the fetal lamb was not different from that observed in the adult lamb. This is because myocardial blood flow and myocardial oxygen extraction were higher in the fetal lamb. As a result, the actual amount of oxygen consumed by the fetal lamb heart was not different from that of the adult lamb heart. High lactate oxidation rates in the fetal heart are also consistent with a substantial oxidative capacity in this setting. Similarly, Lopaschuk et al (1991) demonstrated that 1-day-old rabbit hearts readily oxidize lactate.

In contrast to these findings, a marked difference in oxygen consumption in the fetal and adult guinea pigs hearts has been documented (Rolph and Jones

1983). The difference between these studies may be a result of experimental design or indicate a variation between species considered.

1.6.2. Hormonal Changes in the Newborn Period

Th immediate postnatal period is characterized by an increase in plasma glucagon and a decrease in plasma insulin levels in most species including humans, rats, rabbits, sheep, and pigs (Bassett and Alexander 1971, Blazquez et al 1974). Perinatal increase in plasma glucagon and decrease in plasma insulin has been suggested to be the result of the stress of birth and the activation of the sympathetic nervous system (Girard et al 1973, Padbury et al 1981). Catecholamines stimulate glucagon release and effectively inhibit insulin secretion in the fetus (Girard et al 1974, Girard and Sperling 1983). This hormonal environment is persistent throughout the suckling period, and is thought to be related to the high fat and low carbohydrate content of the diet that is consumed by the newborn.

In rats, the levels of thyroid hormones (T3 and T4) are low at birth and increase markedly within the first 2 weeks (Fisher et al 1977, Walker et al 1980) reaching substantial levels before weaning. The importance of increased thyroid hormones and the effect on energy substrate metabolism in the immediate newborn period remains to be determined.

1.6.3. Subcellular Changes in the Control of Energy Metabolism of the Newborn Heart

1.6.3.1. Carbohydrate Metabolism

The transporter mechanisms for glucose and lactate are unique in the newborn period. During fetal life, glucose uptake is regulated by a low affinity membrane transporter, GLUT-1. The expression of this basal glucose transporter is regulated mainly by plasma glucose levels (Shroeder et al 1997). Shortly after birth, the glucose transporter protein switch from GLUT-1 to the GLUT-4 isoform (Postic et al 1994). The dominant isoform in the adult heart is GLUT-4.

Phosphofructokinase (PFK) is the rate-limiting enzyme of glycolysis (Figure 1.3). It exists in two isoforms, PFK_1 and PFK_2. PFK_1 catalyses the phosphorylation of fructose-6-phosphate to form fructose-1,6-bisphosphate. PFK_2 phosphorylates fructose-6-phosphate to form fructose-2,6-bisphosphate. Fructose-2,6-bisphosphate activates PFK_1 and increases glycolytic flux. Typically, PFK activity is regulated in cardiac tissue by cytosolic ATP and citrate concentrations to prevent any excessive flux of glycolysis and uncoupling of the glycolysis/glucose oxidation ratio. However, there is evidence in the fetal heart that PFK is less sensitive to these inhibitory regulators and more sensitive to fructose-2,6-bisphosphate activation (Bristow et al 1987).

Lactate influx and efflux from the myocardial cell is controlled by the monocarboxylate co-transporter (Poole and Halestrap 1993). The function of the co-transporter is dependent on a trans-membrane H^+ gradient. This is an important feature that the fetal heart exploits to its advantage. Studies from our laboratory showed that the rates of glycolysis and glucose oxidation were markedly different in 1-day-old hearts (Lopaschuk et al 1991). The ratio of glycolysis to glucose oxidation is 46:1 in 1-day-old hearts and 10:1 in 7-day-old hearts. In theory, this should result in a decrease in the intracellular pH. In other words, the uncoupling that was observed in experimental models is useful for the fetal and newborn heart that enables an effective lactate influx into the myocardial cell.

When lactate enters the cytosol, it undergoes reversible oxidation to form pyruvate. This reaction requires NADH as an electron donor and is catalyzed by lactate dehydrogenase (LDH). LDH is a tetrameric complex comprised of varying combinations of the two monomers designated LDH-H (predominant in the heart) and LDH-M (predominant in skeletal muscle).

Studies from the early 1970's in guinea pig suggested that the LDH-H/LDH-M ratio was low in the fetal heart and rise in the first month after birth (Barnie and Harris 1977). Overall myocardial LDH activity has also been shown to be higher in mature than in fetal sheep (Ohtsuka and Gilbert 1995).

Pyruvate decarboxylation is the key irreversible step in carbohydrate oxidation and is catalyzed by pyruvate dehydrogenase (PDH), a multi-enzyme complex located within the mitochondrial membrane. PDH activity is regulated by two regulatory enzymes, pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP). PDK and PDP together catalyze a phosphorylation-dephosphorylation cycle as shown in Figure 1.4. Based on glucose oxidation measurements, it has been suggested that flux through PDH is low in the early newborn period and did not increase until after weaning. In this study we analyzed the activity of PDH and glucose oxidation in parallel and demonstrated that the newborn rapidly acquired the capability of oxidizing glucose but glucose oxidation is inhibited due to the increase in fatty acid oxidation (discussed in Chapter 3).

Table 1.3 summarizes the information related to carbohydrate metabolism in fetal, newborn and adult myocardium.

1.6.3.2. Fatty Acid Metabolism

Fatty acid oxidation in the newborn heart matures rapidly following birth (Werner et al 1989, Lopaschuk and Spafford 1990). For example, in 1-day old rabbit hearts, fatty acid oxidation rates are very low, and contribute less than 10% to overall ATP production (Lopaschuk and Spafford 1990). However, by 7-days of age fatty acid oxidation dramatically increases and becomes the predominant source of energy production. What is responsible for this increase in fatty acid oxidation has not been completely delineated, although an increase in mitochondrial uptake of fatty acids has been implicated in this process (Girard 1992, Lopaschuk et al 1994b, Brown et al 1995). The transport of fatty acids into the mitochondria is primarily controlled at the level of CPT I (McGarry and Foster 1980). As discussed earlier, CPT I is inhibited by malonyl CoA and stimulated by L-carnitine.

As discussed earlier, the increase in fatty acid oxidation is attributed to a decrease in myocardial malonyl CoA levels following birth, resulting in a decrease in malonyl CoA inhibition of CPT I. In 1-day old rabbit hearts, malonyl CoA levels are very high, but decrease dramatically by 7-days following birth (Lopaschuk et al 1994b, Dyck et al 1998, Makinde et al 1997). These changes in malonyl CoA can be explained by alterations in the control of both malonyl CoA synthesis and degradation (Lopaschuk et al 1994a, Lopaschuk et al 1994b, Dyck et al 1998, Makinde et al 1997).

Malonyl CoA is synthesized in the heart by ACC, the activity of which decreases in the heart following birth (Lopaschuk et al 1994a). ACC is in turn phosphorylated and inactivated by AMPK (Hardie et al 1997), the activity and expression of which increases in the heart following birth (Makinde et al 1997). The relative importance of changes in MCD and ACC control of malonyl CoA versus changes in L-carnitine control of CPT I or alterations in CPT I activity/expression to the increase in fatty acid oxidation in the newborn heart is not clear.

1.6.3.3. Role of Carnitine in Perinatal Metabolism

Carnitine (gamma-trimethyl-beta-hydroxy-butyrobetaine) is a quaternary amine that is involved in mammalian intermediary metabolism (Bremer 1983). Carnitine is synthesized by the liver and to a lesser degree by the kidneys from the amino acid precursors lysine and methionine (Bremer 1983). Carnitine acts as a cofactor in the mechanism by which fatty acids are transported for mitochondrial β-oxidation and for the regeneration of free coenzyme A from various acyl CoA intermediates. Carnitine also regulates the concentration of free CoA in the mitochondrial matrix by removing excess acyl groups (Ramsay 1994). This effect of carnitine is important for the regulation of carbohydrate metabolism (Broderick et al 1993). Stimulation of pyruvate dehydrogenase complex by the export of acetyl CoA from the mitochondria appears to account for the beneficial effects of L-carnitine in treating certain cardiac diseases (Broderick et al 1993, Martin et al 2000).

In healthy adults, approximately 16-20 mg/day (100-125 μmol/day) of L-carnitine may be synthesized endogenously (Rudman et al 1977). Total body supply of carnitine derives in part from dietary sources (Melegh et al 1990). Carnitine reserves of the body are almost completely pooled in the muscles (Engel and Rebouche 1984). Myocardial carnitine levels also greatly exceed plasma levels as the heart can extract carnitine against a sixty-fold concentration gradient. Myocardial uptake of carnitine occurs by means of a specific sodium-dependent carnitine transporter (Tamai et al 1998, Nezu et al 1999).

Earlier studies in this area indicated that the carnitine of neonatal tissues is derived primarily from carnitine synthesized by the maternal liver (Robles-Valdes et al 1976). As gestation proceeds, the blood carnitine concentration gradually decreases in pregnant women (Hahn et al 1977). The total and free carnitine concentrations are higher in umbilical blood and in plasma of infants at birth than that found in the mother (Battistella et al 1980, Novak et al 1981). Supplementation of the infant with carnitine from the mother continues during the suckling period. However, as shown by Davis (1989), the rat fetus contributes approximately 50% to its own carnitine stores by endogenous synthesis.

A change in myocardial carnitine concentrations has been suggested to play a role in the physiological switch of the newborn heart from using carbohydrates to using fatty acids (Brown et al 1995). In that study, increase in the M- (low affinity for carnitine) versus L-isoform (high affinity for carnitine) has been considered with the increase in total carnitine pool in the newborn heart. The relative importance of carnitine and malonyl CoA in the maturation of fatty acid oxidation is examined as a part of this thesis (Chapter 3).

1.6.4. Ischemia-Reperfusion in Experimental Models and Clinical Applications

The protection of neonatal hearts in the setting of an open heart surgery to correct congenital heart defects remains a fundamental clinical problem. Current methods of myocardial protection during adult open heart surgery include a variety of cardioplegic techniques. Myocardial protection techniques used in adult hearts are generally extended to pediatric heart surgery. Mortality rates from open heart surgery are higher in neonates than adults, which had been attributed to inadequate myocardial protection during ischemia (Bull et al 1984, Hammon et al 1987, del Nido et al 1988, Imura et al 2001).

On the other hand, available laboratory data suggests that developing mammalian hearts are more resistant to the damaging effects of ischemic insults than adult hearts (Jonas 1998, Matherne et al 1996, Baker et al 1997). Many pathways have been suggested to be responsible for this increased tolerance of the immature heart to an ischemic insult, including increased NO production, K_{ATP} channels, and vascular resistance. The reasons for this discrepancy between the clinical observations and experimental studies are currently not known. A very important difference between experimental studies and the clinical setting is the absence of added fatty acids in most of the studies examining ischemic tolerance in *ex vivo* newborn hearts. In fact, high levels of fatty acids have been reported post-surgery in infants undergoing cardiac bypass operation to correct congenital heart defects (Lopaschuk et al 1994). Moreover, in both mature (Liedtke et al 1983, Lopaschuk et al 1990) and immature hearts (Lopaschuk et al 1994) high levels of fatty acids have been shown to affect the recovery of the heart after ischemia. Therefore, the comparison of the experimental data and the clinical situation is difficult. Metabolic parameters and mechanical function following ischemia in the newborn heart has also been examined in our laboratory where the perfusate included 1.2 mM palmitate in order to mimic the high concentrations of free fatty acids seen in the clinical setting (Itoi et al 1993, Itoi and Lopaschuk 1996). In these studies, increases in extracellular Ca^{+2} resulted in an increase in the post-ischemic recovery and ATP production in the immature heart (Itoi and Lopaschuk 1996). These effects of Ca^{+2} in the immature heart differ dramatically from what has been observed in mature hearts. In adult rat hearts, high Ca^{+2} concentrations were reported to be detrimental (Broderick et al 1993).

Adult and immature hearts also differ in their responsiveness to stimulation of PDC activity. In the presence of high fatty acids, PDC activity is low due to an increased intramitochondrial acetyl CoA /CoA ratio derived from β-oxidation. In adult hearts, pharmacological stimulation of PDC by dichloroacetate (DCA) is associated with a significant improvement of mechanical recovery following ischemia (Lopaschuk et al 1993). However, the effects of DCA on functional recovery of immature hearts subjected to ischemia are less dramatic as shown by Itoi et al (1993) and Itoi and Lopaschuk (1996). Therefore, a better recovery post-ischemia has been suggested to be associated with other reasons than stimulation of glucose oxidation (Itoi and Lopaschuk 1996).

One such mechanism might be an overall increase in oxidative metabolism as opposed to specifically increasing carbohydrate oxidation. In the study by Itoi and Lopaschuk (1996), an overall increase in palmitate, lactate, and glucose oxidation was demonstrated when extracellular Ca^{+2} was increased. Ca^{+2} has also been shown to increase mitochondrial volume and stimulate palmitate oxidation in isolated heart mitochondria (Halestrap et al 1987). Moreover, an increase in intramitochondrial matrix volume has been suggested to be the underlying mechanism of the beneficial effects of K_{ATP} channel openers in the adaptation of the heart to chronic hypoxia (Eels et al 2000).

Whether the newborn heart is more resistant to the ischemic damage in the post-operative setting compared to adult heart remains unresolved. However, studies suggest that the overall increase in energy-producing pathways improve the post-ischemic recovery in the newborn heart. If indeed immature heart is more resistant to the detrimental effects of ischemia than the adult heart, the increase in fatty acid oxidation might also increase the sensitivity of the immature heart to ischemic damage.

1.7. Metabolic Effects of Adiponectin

1.7.1. Discovery of a New Adipokine

The cDNA encoding adiponectin was first reported by Scherer's group in 1995 (Scherer et al 1995). The mRNA was induced over 100-fold during differentiation of preadipocytes and was expressed exclusively in adipocytes. The transcript encoded a hydrophilic protein with a predicted size of 29 kDa. The protein was called adipocyte complement-related protein of 30 kDa (Acrp30) based on its resemblance to complement factor C1q, and on its size by SDS-PAGE electrophoresis (Scherer et al 1995).

Adiponectin is also called AdipoQ (Hu et al 1996), apM1 (adipose most abundant gene transcript 1) (Maeda et al 1996), adiponectin (Ouchi et al 1999, Arita et al 1999), and GBP28 (gelatin-binding protein 28) (Nakano et al 1999). All of these names are currently used in the literature. In this study, we refer to the protein as adiponectin.

1.7.2. Structure of Adiponectin

The comparison of the primary amino acid sequence of adiponectin reveals a close similarity in human, rat, rhesus monkey, cow and mouse adiponectin sequences (Table 1.4). All of the adiponectin homologues have similar structure comprising four main domains: a cleaved amino-terminal signal sequence, a species-specific non-homologous region, a collagenous region, and a globular segment at the carboxy terminus (Figure 1.5).

The three-dimensional structure of the C-terminal globular domain of adiponectin is similar to that of tumor necrosis factor α (TNFα) even though there is no homology at the primary sequence level (Shapiro and Scherer 1998).

Adiponectin shares squence homology with a family of proteins containing a characteristic C-terminal complement factor C1q-like globular domain. Protein cleavage is a common feature in the activation of the complement cascade. For instance, precerebellin, another protein that contains a C1q-like globular domain, is proteolytically cleaved into active cerebellin peptide (Kishore and Reid 1999). A similar feature was proposed for adiponectin by Fruebis et al (2001). These authors showed that bacterially expressed and purified C-terminal globular domain of adiponectin (gAd) is also physiologically active and stimulates free fatty acid oxidation in muscle, as well as induces weight reduction in mice. The protease cleavage site on adiponectin is illustrated in Figure 1.5. Additional studies with either the full-legth or the gAd will be discussed in detail in the following sections.

The collagenous domain of adiponectin contains Gly-X-Y or Gly-X-Pro consensus sequences. These sequences are found in proteins that form collagenous triple helices (Berg et al 2002). There are also several conserved prolines and lysines within the collagenous domain which have been suggested to be hydroxylated and form stable collagen triple helices (Berg et al 2002).

The basic form of adiponectin is a homotrimer (Shapiro and Scherer 1998, Berg et al 2002). The interactions between the trimers of the adiponectin form oligomers that are also found in the plasma. It has been reported that two to six trimers can assemble which results in a concentration of 5-20 nM in the plasma (Scherer et al 1995). Recently, Scherer's group demonstrated that adiponectin existed in two discrete stable complexes in the serum, as a hexamer (low molecular weight form, ~180 kDa) and a higher order complex between 12-18 subunits (high molecular weight form, greater than 400 kDa) (Pajvani et al 2003). The authors also showed that the high molecular weight species of adiponectin is susceptible to acidic pH degradation, resulting in the formation of the hexameric low molecular weight form. Addition of a reducing agent further reduced the hexamer to even lower molecular weight species consistent with the molecular weight of the trimer (~90 kDa). These results indicate the importance of disulphide bonds in the formation of high molecular weight species of the adiponectin (Pajvani et al 2003).

A cysteine residue in the N-terminal region, immediately preceeding the collagenous domain is conserved among the structural homologues of adiponectin (Pajvani et al 2003). Cys39 was suggested to be responsible for the disulfide bond formation, since the only other cysteine, Cys155, did not appear to be properly spaced for interaction with other chains and disulphide bonding in an oligomeric structure (Pajvani et al 2003). Indeed, substitution of Cys39 with a serine residue by site-directed mutagenesis resulted in the formation of a mutant adiponectin that does not form higher order structures (Pajvani et al 2003). The authors next transfected HEK 293-T cells with both wild-type and Cys39 mutated expression constructs. Interestingly, the mutated protein was secreted more efficiently than the wild-type protein. In addition, a proteolytic cleavage product of ~26 kDa was found in the extracellular fraction of the cells that produce the mutated protein. These results demonstrated that the mutant, trimeric form of adiponectin is more susceptible to specific cleavage in cell culture, and that the cleavage process takes place after it is secreted. The same proteolytic cleavage product is seen in reduced wild-type adiponectin, and is more active than the wild-type protein with respect to lowering serum glucose subsequent to intravenous administration.

A working model for adiponectin action was proposed by Scherer's group: The high molecular weight adiponectin complexes circulating in the serum represent a pool that can be activated. Activation is triggered by metabolic stimuli which may trigger the dissociation of the high order complex to a transient appearance of the bioactive dimer.

Several other groups reported post-translational modifications that may be important for adiponectin function. Wang et al (2002) substituted four of the conserved lysine residues in the collagenous domain with arginines. This mutation resulted in the ablation of hydroxylation and glycosylation of these residues. This mutation resulted in an inactive protein that failed to inhibit glucose output by hepaocytes. However, as discussed earlier, the collagenous domain of the protein is also involved in the formation of the higher order structures of adiponectin. Another post-translational modification has been reported by Sato et al (2001). The physiological importance of the post-translational modifications of adiponectin and/or the formation of the stable higher order structures through disulphide bonds requires further investigation.

1.7.3. Metabolic Effects of Adiponectin

1.7.3.1. Metabolic Disorders and Other Modifications that Affect Adiponectin Levels

Several lines of evidence suggest that adiponectin plays a role in the regulation of metabolism. Hu et al (1996) showed that the levels of transcripts for adiponectin in white adipose tissue were lower in ob/ob mice than in wild-type mice. Arita et al (1999) reported that adiponectin was abundantly present in human plasma. Plasma concentrations of most proteins produced by the adipose tissue are increased in obesity because of an increase in the total body fat mass. For instance, the plasma concentration of leptin is increased when BMI is increased (Hosoda et al 1996). In the study by Arita et al (1999), the plasma levels of adiponectin in obesity were lower than those in non-obese subjects. Also, plasma adiponectin levels in men are significantly lower than in women among both obese, and non-obese subjects. In another large-scale epidemiological study, Weyer et al (2001) confirmed the findings that obesity and type 2 diabetes are associated with low plasma adiponectin concentrations. The Pima Indians of Arizona were used in that study, because they have among the highest reported prevalence rates of obesity, insulin resistance, and type 2 diabetes in the world (Weyer et al 2001). The mean plasma adiponectin concentration is lower in non-diabetic Pima Indians than in Caucasians and in subjects with impaired glucose tolerance and diabetes compared with those with normal glucose tolerance. The same group later reported that low plasma adiponectin concentrations were associated with a high basal and decreased insulin-stimulated tyrosine phosphorylation of the insulin receptor in skeletal muscle (Stephan et al 2002).

Similar results were obtained in rhesus monkeys, a species genetically predisposed to develop insulin resistance (Hotta et al 2001). In both studies, insulin sensitivity was more strongly associated with high adiponectin levels than body fat mass.

Two independent groups mapped the metabolic syndrome X (Kissebah et al 2000, Comuzzi et al 2001) and diabetes (Vionnet et al 2000) susceptibilty locus on 3q27 where the adiponectin gene is located (Das et al 2001, Takahashi 2000). Several studies showed that variations on the adiponectin gene results in lower adiponectin concentrations, type 2 diabetes, obesity, and insulin resistance (reviewed in Ukkola and Santaniemi 2002). For example, mutations affecting the globular head domain (especially isoleucine to threonine mutation at position 164, I164T) were shown to result in lower level of adiponectin and some features of metabolic syndrome such as hypertension, hyperlipidemia, diabetes, and atherosclerosis compared to control Japanese patients (Kondo et al 2002).

Adiponectin levels do not change in a diurnal or postprandial fashion (Motoshima et al 2002, Berg et al 2001). Recent publications reported significant increases in plasma adiponectin with weight loss in mice (Berg et al 2001), and in human subjects (Hotta et al 2000, Yang et al 2001). This further supports the conclusion that circulating adiponectin levels are not proportional to overall body fat mass. However, exercise training, another common intervention for the treatment of insulin resistance, does not affect plasma levels of adiponectin (Hulver et al 2002).

1.7.3.2. The Effects of Adiponectin Therapy

Yamauchi et al (2001) examined the effects of adiponectin administration in several mouse models. In wild-type mice on a high-fat diet, ob/ob mice and KK^y obese diabetic mice, long-term adiponectin infusion improved insulin sensitivity. These authors also reported that adiponectin had beneficial effects on lipid metabolism. They used a lipoatrophic mice model, and found that adiponectin increased β-oxidation in muscle, and decreased triglycerides in liver and muscle.

Plasma triglycerides and free fatty acids were also decreased with adiponectin treatment.

Scherer's group initially focused on the effects of adiponectin on glucose metabolism. They injected purified adiponectin into the intraperitoneal cavity of wild-type C57B1/6J, ob/ob, NOD (non-obese diabetic) or streptozotocin-diabetic mice. This treatment resulted in an elevation in circulating adiponectin levels and a transient decrease in glucose levels. Importantly, all the mouse experiments were performed near basal conditions where insulin levels were low. Under these conditions, blood glucose levels are primarily determined by hepatic glucose output. Therefore, the drop in glucose following adiponectin injection was suggested to be a result of the hepatic effect of adiponectin. The authors verified this conclusion by measuring the effect of adiponectin on glucose output from isolated primary rat hepatocytes. Adiponectin increased the sensitivity of adipocytes to the effects of insulin on glucose production. In other words, insulin suppressed glucose production in these cells at lower concentrations when adiponectin was present compared to the insulin-only condition.

Insulin levels remained unchanged in the study by Berg et al (2001) despite a significant decrease in glycemia in response to adiponectin. In another study by Fruebis et al (2001) the authors reported a moderate increase in plasma insulin concentrations in response to the globular fragment of adiponectin. Combs et al (2001) infused purified recombinant adiponectin in conscious mice during a pancreatic euglycemic clamp. They created a modest (~ 40% increase compared to the basal insulin levels) increase in plasma insulin in these mice. Thus, they examined the effects of adiponectin in the presence of similar steady-state insulinemia and normoglycemia. They also infused somatostatin to prevent changes in glucagon during the clamp procedure. In the presence of a pancreatic clamp, infusion of adiponectin led to a marked suppression of endogenous glucose production in mice. The authors also reported a decrease in glucose-6-

phosphatase activity by adiponectin infusion compared with vehicle infusion (Combs et al 2001).

Fruebis et al (2001) prepared a fragment of the full length adiponectin by trypsinization, and partially purified the resulting globular trimer (gAd). They reported that gAd was present in human plasma. They investigated the physiological roles of full-length adiponectin and gAd by feeding normal C57BL/6J mice a high-fat/sucrose meal and followed the postprandial response of plasma free fatty acids, glucose, and triglycerides. Saline-injected animals showed the expected rise in all these parameters after the meal. However, injection of gAd at the time of gavage and two times thereafter significantly decreased the levels of plasma free fatty acids, glucose, and triglycerides. Interestingly, only small and transient effects were observed in mice injected with full-length adiponectin. They next investigated the effect of gAd on muscle fatty acid oxidation in isolated EDL and soleus muscle using oleate as substrate, and demonstrated that gAd significantly increased oleate oxidation in both muscle types (Fruebis et al 2001). Furthermore, the authors reported that chronic administration of gAd prevented high-fat diet-induced weight gain.

Scherer's group also tested the effects of bacterially produced gAd on glucose production on isolated hepatocytes. gAd had no effect on glucose production in this model (Berg et al 2001).

As noted at the beginning of this section, Yamauchi et al (2001) reported beneficial effects of adiponectin in several mice models. In addition to the effects by gAd used by Fruebis et al (2001) they saw effects with the full-length bacterial product. However, the potency of the full-length adiponectin was lower.

It is important to note that different groups have prepared different forms of adiponectin using either bacterial or mammalian expression systems. Further experiments are needed to determine whether these findings reflect different activities between adiponectin and gAd or these differences relate to eucaryotic cell post-translational modifications necessary for certain adiponectin effects but lacking in bacterially expressed protein.

Recently, Yamauchi et al demonstrated that both gAd and full-length adiponectin stimulated AMPK in skeletal muscle (Yamauchi et al 2002). The authors showed that this activation increased the phosphorylation of ACC, fatty acid oxidation, glucose uptake and lactate production in C2C12 myocytes. When administrated *in vivo*, both full-length adiponectin and gAd stimulated AMPK and increased the phosphorylation of ACC in mouse soleus muscle (Yamauchi et al 2002). Moreover, stimulation of AMPK with full-length adiponectin reduced gluconeogenesis in the liver and plasma glucose in the same study (Yamauchi et al 2002). In agreement with these findings, Tomas et al (2002) reported that gAd stimulates fatty acid oxidation in skeletal muscle by activating AMPK and inactivating ACC.

While we were preparing this thesis, Yamauchi et al (2003) cloned complementary DNAs encoding adiponectin receptors AdipoR1 and AdipoR2. The authors showed that AdipoR1 is abundantly expressed in skeletal muscle and AdipoR2 is the predominant form in the liver. They also suggested that AdipoR1 is a high-affinity receptor for gAd and a low-affinity receptor for full-length adiponectin. This paper will be discussed in more detail in Chapter 5.

1.7.3.3. Lessons From Gene Disruption Experiments

Three groups reported slightly different phenotypes in adiponectin knockout mice: Kubota et al (2002) observed moderate insulin resistance in mice that were heterozygous for the adiponectin gene. This effect was more pronounced in mice lacking both copies of the gene.

Maeda et al (2002) also produced adiponectin knockout mice, but did not observe evidence of insulin resistance when adiponectin knockout mice were fed a regular chow. However, they demonstrated that feeding the knockout mice a high fat/high sucrose diet for two weeks induced insulin resistance. They concluded that only when nutrition was overloaded did knockout mice develop severe insulin resistance. Without over-nutrition, loss of adiponectin was not sufficient to induce overt metabolic disorders. Similar results came from another group. Ma et al (2002) did not observe insulin resistance in their model of knockout mice. Interestingly, these authors demonstrated increased β-oxidation in the adiponectin knockout mice. They suggested that the overall phenotype of adiponectin knockout could be the result of the activation or overexpression of compensatory biochemical pathways. They also suggested that in the experiments in which recombinant adiponectin was injected into animals this adiponectin might have acted as a dominant negative molecule that blocked the normal action of native adiponectin *in vivo*. Clearly, additional experiments are needed to address this issue.

1.7.3.4. Hormonal Regulation of Adiponectin Gene Expression

A number of hormones causing insulin resistance *in vivo* and *in vitro* have recently been tested on their ability to alter adiponectin gene expression (Fasshauer et al 2002). Such an interaction was extremely important for our studies since dramatic alterations occur in the newborn period in the plasma levels of several hormones including insulin and glucagon. To investigate the regulation of adiponectin gene expression *in vitro*, Fasshauer et al (2002) incubated 3T3-L1 adipocytes with insulin, TNFα, dexamethasone, angiotensin (AT) 2, growth hormone (GH), or triiodothyronine (T3). Treatment of these cells with insulin decreased adiponectin mRNA by 85% compared to untreated controls. Similarly, both TNFα and dexamethasone significantly decreased adiponectin gene expression whereas T3, AT2, and GH did not affect adiponectin mRNA expression. The authors showed that the suppressant effect of insulin, TNFα, and dexamethasone on gene expression was reversible, since removal of the hormones from the medium for 24 hours fully restored adiponectin mRNA levels to control levels. Furthermore, by using specific inhibitors they showed evidence that insulin exerts its effect at least partly via multiple signaling pathways involving p44/42 MAP kinase, PI 3-kinase, and p70S6 kinase. The same group also reported that isoproterenol inhibited adiponectin gene expression in 3T3-L1 adipocytes (Fasshauer et al 2001). They incubated the cells with H-89, a selective inhibitor of protein kinase A (PKA), and almost completely reversed the inhibitory effect of isoproterenol on adiponectin gene expression. Based on these results, the authors concluded that β-adrenergic stimulation with isoproterenol reduces adiponectin gene expression via adenylyl cyclase and PKA. Similar results came from Zhang et al (2002). These authors demonstrated that CL316,243, a selective β3-agonist decreased adiponectin mRNA expression in both white and brown adipose tissues of rats. This decrease failed to result in a decrase in the plasma levels of adiponectin. However, chronic administration of CL316,243 by osmotic minipump enhanced adiponectin mRNA levels in the white adipose tissue of rats. Serum adiponectin concentrations were also elevated. Based on these results, the authors concluded that regulation of adiponectin demonstrated a unique short- and long-term pattern. In aggrement with both of these studies, isoproterenol, another β-adrenergic agonist, BRL37344, and cAMP were all shown to decrease adiponectin mRNA expression both in mouse cells, and *in vivo* (Delporte et al 2002). In accordance with the *in vitro* data, hyperinsulinemia in humans has been shown to decrease plasma levels of adiponectin in human subjects *in vivo* (Möhlig et al 2002).

1.8. The Purpose of this Thesis

The newborn heart of most species is unique in that a transition occurs at birth from using carbohydrates to using fatty acids as the primary source of energy. Although complicated by the contribution of many factors such as hormonal and nutritional changes, the newborn heart still is a very useful model for studying the subcellular regulation of fatty acid metabolism. Also, the rapid maturation of fatty acid oxidation following birth has been known to decrease the ability of the heart to withstand an ischemic episode such as that which occurs during cardiac surgery to correct congenital heart defects. The identification of the steps involved in the metabolism of fatty acids will provide useful information that can be used in the development of new drugs.

1.9. Specific Objectives of the Thesis

1. To determine the relative contribution of CPT I isoform expression, myocardial L-carnitine content and the kinetics of CPT I to the maturation of fatty acid oxidation in the newborn heart. The following questions were asked:
    a. Does CPT I have the substrates and the capacity to process fatty acids to the same degree throughout the newborn period?
    b. Does CPT I have the same affinity for carnitine in the newborn period?
    c. Does the sensitivity of CPT I for malonyl CoA change in the newborn period?
    d. Does isoform expression change in the newborn period? If it does, is the change parallel with the increase in fatty acid oxidation?
2. To determine the regulatory role of malonyl CoA when the myocardium is exposed to higher concentrations of fatty acids. The following questions were asked:
    a. Do the rates of fatty acid oxidation increase as a response to increased fatty acid supply?
    b. Do the tissue levels of malonyl CoA further decrease to relieve the inhibition on CPT I when the myocardium is exposed to higher concentrations of fatty acids?
    c. How are the enzymes that are involved in the regulation of tissue levels of malonyl CoA contribute to the increase in fatty acid oxidation when the myocardium is exposed to higher concentrations of fatty acids?
3. To determine the initial signal for the subcellular changes that result in increased fatty acid oxidation in the immediate newborn heart. We examined the potential involvement of adiponectin as the initial signal for the increase in fatty acid oxidation. The following questions were asked;

   a. Do the plasma levels of adiponectin change in the newborn period?
   b. Does adiponectin/gAd increase fatty acid oxidation in 1-day-old heart?
   c. Does adiponectin induce the subcellular metabolic changes that are already identified in the newborn period?
4. To examine the effects of gAd on fatty acid and glucose metabolism in 7- day old hearts. The following questions were asked:
   a. Does gAd have an effect on cardiac glucose and fatty acid oxidation in 7-day-old hearts?
   b. How are the primary enzymes that are involved in the metabolic pathways affected by gAd treatment?

1.10. Experimental Approach

In this thesis, New Zealand White rabbits were chosen as an experimental model. Our laboratory has extensive experience with this species. We also had to consider the size of the 1-day-old rabbit heart.

We examined the relative importance of malonyl CoA and carnitine on the maturation of fatty acid oxidation in intact and purified mitochondria. It was very important to have an intact mitochondrial fraction, since other acyltransferases such as carnitine palmitoyltransferase II (CPT II) of the mitochondrial inner membrane, or carnitine acylcarnitine translocase (CAT) are not inhibitable by malonyl CoA. In order to examine the characteristics of CPT I, we had to perform our experiments in pure and intact mitochondria.

Isolated heart perfusion is a very powerful tool for studying energy metabolism. It allowed us to examine the metabolic changes as well as to simultaneously follow the work that was performed by the heart.

Two different perfusion techniques (isolated working heart and Langendorff perfusion) were used in this study because the *foramen ovale* closes in small animals within 3-4 days after birth. The *foramen ovale* is the opening in the septum between the right and the left atria in the fetal heart. This opening provides a bypass for blood that would otherwise flow to the fetal lungs. Since the perfusion techniques were different, we could not directly compare the metabolic parameters of the rabbit hearts between 1-day and 7-day hearts. Instead, we examined the effects of adiponectin within the same age group.

Finally, the ability to compare the metabolic parameters with the activity and expression of the enzymes that are involved in the intermediary metabolism was important to understand the intracellular regulation of the energy metabolism in the newborn period.

Table 1.1 Conditions or treatments that modify malonyl CoA levels and fatty acid oxidation rates in the heart

Condition/ treatment	malonyl CoA levels	AMPK activity	ACC	MCD	Fatty Acid Oxidation
Fetal to Newborn Transition	↓ [1,2]	↑ [3]	↓ [1,2]	↑ [1]	↑ [1]
Adiponectin	ns	↑ [4]	↓[4]	ns	↑ [4]
Cardiac Work	↓ [5,6]	nc [6]	nc [5,6]	↑[5]	↑ [5]
Diabetes	↓ [7,8]	nc [7]	nc [7]	↑ [7]	↑[7]
Ischemia/ Reperfusion	↓ [1,9,10]	↑ [9]	↓ [1,9]	nc [1]	↑ [1,9,10, 11,12,13]
Hypertrophy	ns	↑[14]	ns	↓ [15]	↓ [15]
PPARα Activation	ns	ns	ns	↑ [15]	↑ [16]
PPAR KO	↑[17]	nc [17]	nc [17]	↓ [17]	↓ [17]

(nc, no change; ns, not studied)

1. Dyck 1998
2. Lopaschuk 1994
3. Makinde 1998
4. Yamauchi 2002
5. Goodwin 1999
6. Hall 1996
7. Sakamoto 2000
8. Gamble 1994
9. Kudo 1995
10. Saddik 1993
11. Lerch 1992
12. Liedtke 1988
13. Lopaschuk 1990
14. Tian 2001
15. Young 2001
16. Finck 2002
17. Campbell 2002

AMPK = AMP-activated protein kinase
ACC = Acetyl CoA carboxylase
MCD = Malonyl CoA decarboxylase
PPAR = Peroxysome proliferator-activated receptor
KO = Knockout

Table 1.2 Levels of circulating substrates and hormones in the fetus, newborn and adults*

Parameter	Fetal	Newborn	Adult
Glucose (mM)	~5	~6	~5
Lactate (mM)	~10	~2	~0.5
Fatty acids (mM)	~0.02	~0.3	~0.4
Insulin (μU/ml)	~95	~10	~40
Glucagon (ng/ml)	~0.18	~1.0	~0.11
Triiodothyronine (ng/dl)	~7	~18	~90

*Values are from Medina et al (1985), and Girard et al (1992)

Table 1.3. Summary of the changes related to carbohydrate metabolism in fetal, newborn and mature myocardium*

	Fetal	**Newborn**	**Mature**
Dominant carbohydrate substrate	lactate	glucose	glucose
% total contribution to ATP	40-60	40	10
Dominant glucose transporter	GLUT-1	GLUT-1 GLUT-4	GLUT-4
Enzyme activity (relative to mature heart)			
PFK	high	very high	
LDH-M	high	high	
PDH	high	low	
TCA cycle	low	same	

*Adapted from Makinde et al 1998

Table 1.4. Primary amino acid sequence comparison between species-specific adiponectin proteins*

Species	%Amino acid similarity
Rat	92
Human	83
Cow	91
Monkey	83

*Berg et al 2002

Figure 1.1. Overview of myocardial energy metabolism

Glucose and fatty acids are primary sources for energy production in the adult heart. Following uptake, the major metabolic pathway of glucose is that of glycolysis. The pyruvate derived from glycolysis is either converted to lactate, or transported into the mitochondria where it is decarboxylated and converted to acetyl CoA. Fatty acids are transported into the cytoplasm, and activated to their CoA esters by a fatty-acyl CoA synthase (FACS). The acyl CoA is converted to acylcarnitine by carnitine palmitoyltransferase I (CPT I) translocated across the inner mitochondrial membrane by carnitine acyltranslocase (CAT) and is then converted back to acyl CoA by carnitine palmitoyltransferase II (CPT II). The intramitochondrial acyl CoA then enters the fatty acid β-oxidation spiral. Acetyl CoA derived from oxidation of glucose or β-oxidation of fatty acids enters the Krebs' Cycle. The electron-carrying species $NADH_2$ (produced either in glycolysis or in the Krebs' Cycle) and FADH (produced in Krebs' Cycle) are shuttled through a sequence of transformations called the electron transport chain (ETC). The hydrogen on $NADH_2$ and FADH is transferred to water in the presence of oxygen, and ATP is produced.

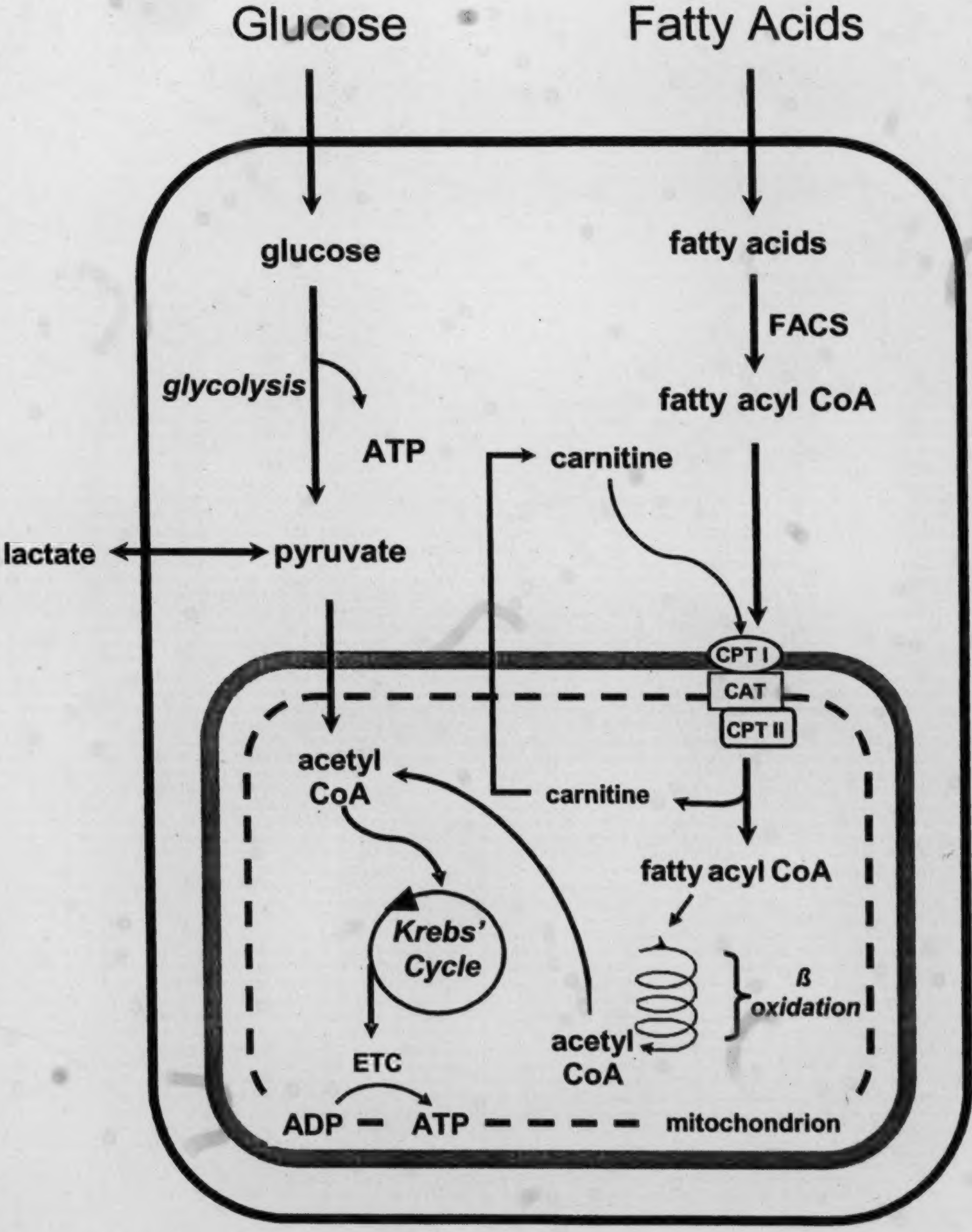
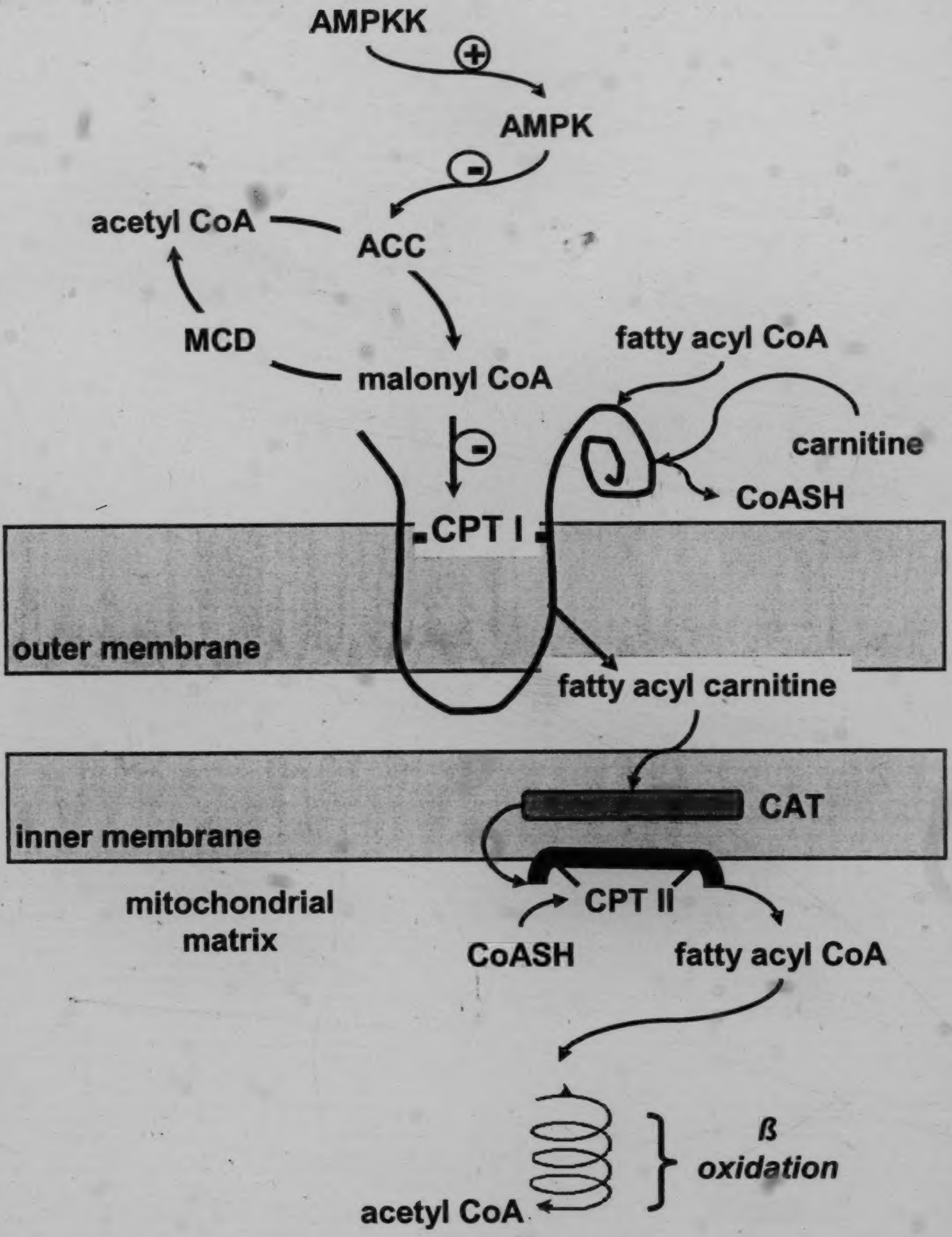

Glucose
Fatty Acids
glucose
fatty acids
FACS
glycolysis
fatty acyl CoA
ATP
carnitine
lactate
pyruvate
CPT I
CAT
CPT II
acetyl CoA
carnitine
fatty acyl CoA
Krebs' Cycle
ß oxidation
acetyl CoA
ETC
ADP
ATP
mitochondrion

Figure 1.2. Regulation of fatty acid oxidation by malonyl CoA

Malonyl CoA is a potent inhibitor of CPT I. Acetyl CoA carboxylase (ACC) in the cytosol synthesizes malonyl CoA from acetyl CoA. 5'-AMP-activated protein kinase (AMPK) phosphorylates and inactivates ACC. AMPK activity is increased by AMPK kinase (AMPKK). Malonyl CoA is degraded by the action of malonyl CoA decarboxylase (MCD).


AMPKK
+
AMPK
-
acetyl CoA
ACC
MCD
malonyl CoA
fatty acyl CoA
carnitine
CoASH
-
CPT I
outer membrane
fatty acyl carnitine
CAT
inner membrane
mitochondrial matrix
CPT II
CoASH
fatty acyl CoA
ß oxidation
acetyl CoA

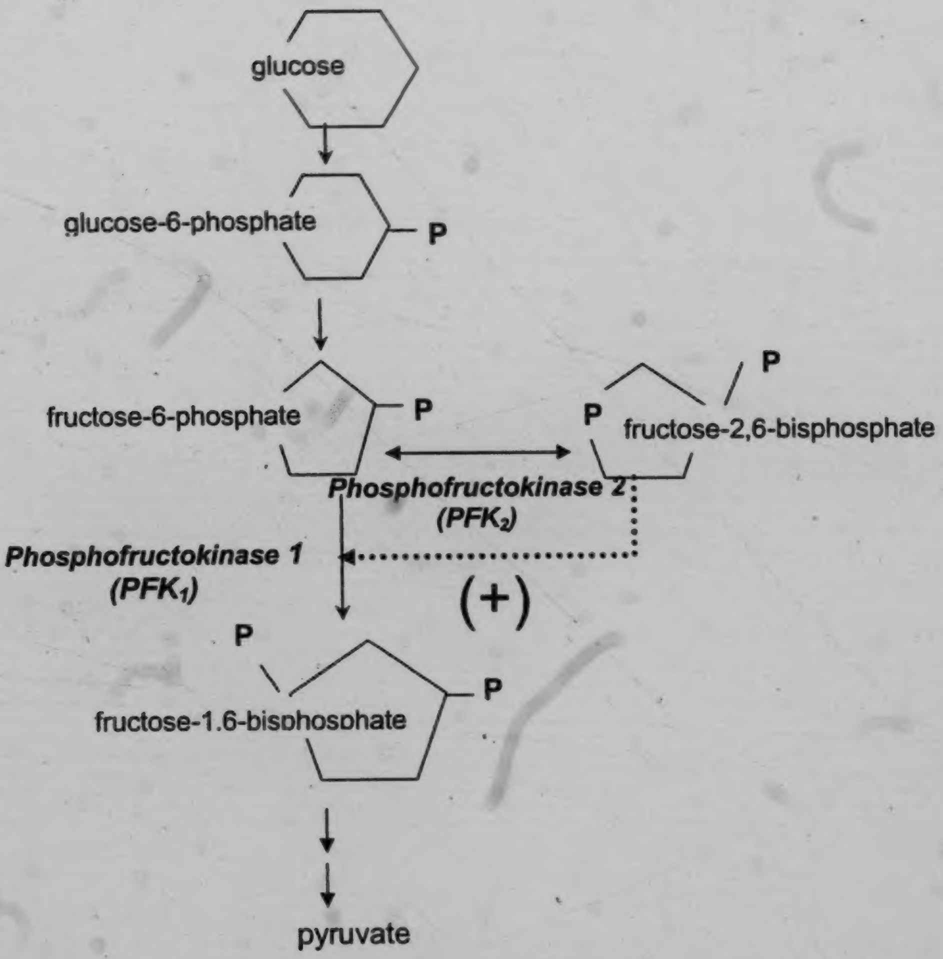

glucose
glucose-6-phosphate
P
fructose-6-phosphate
P
P
P
fructose-2,6-bisphosphate
Phosphofructokinase 2
(PFK₂)
Phosphofructokinase 1
(PFK₁)
(+)
P
P
fructose-1,6-bisphosphate
pyruvate

Figure 1.3. Overview of glycolytic flux

PFK$_1$ catalyses the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate.

PFK$_2$ phosphorylates fructose-6-phosphate to form fructose-2,6-bisphosphate. Fructose-2,6-bisphosphate activates PFK$_1$ and increases glycolytic flux.

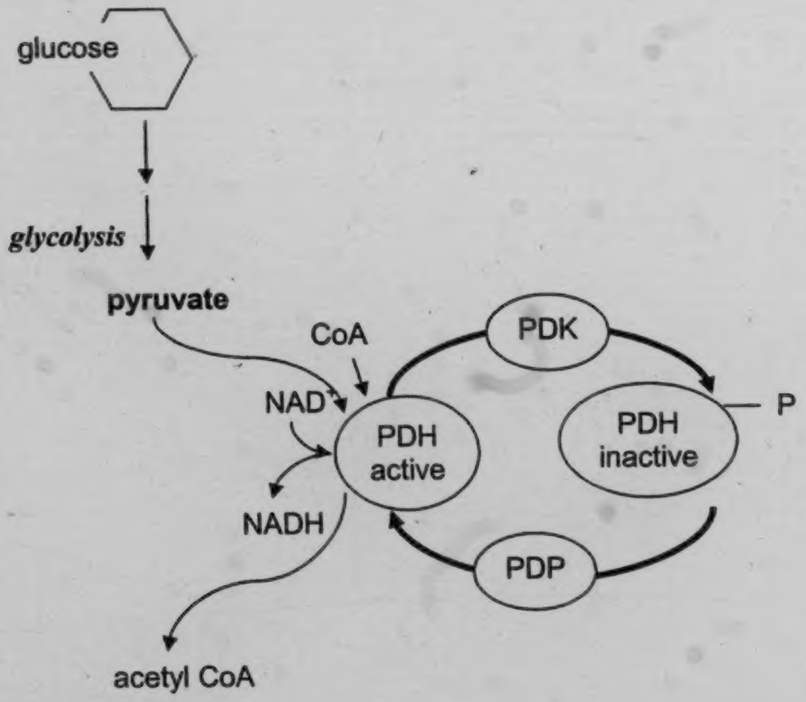

glucose
glycolysis
pyruvate
CoA
NAD⁺
NADH
PDH active
PDK
PDH inactive
P
PDP
acetyl CoA

Figure 1.4. Regulation of pyruvate dehydrogenase by phosphorylation and dephosphorylation

Pyruvate decarboxylation is catalyzed by pyruvate dehydrogenase (PDH), a multi-enzyme complex located within the mitochondrial membrane.

PDH activity is regulated by two regulatory enzymes, pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP).

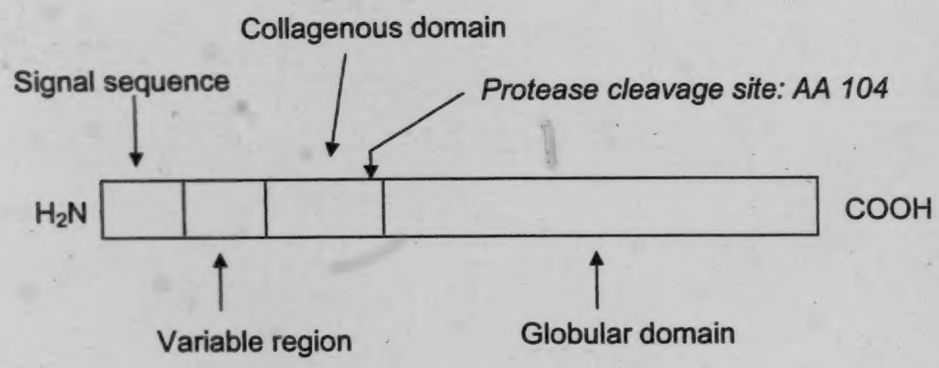

Collagenous domain
Signal sequence
Protease cleavage site: AA 104
H2N
COOH
Variable region
Globular domain

Figure 1.5. Structural features of adiponectin

Adiponectin has four domains: signal sequence, species-specific variable domain and globular trimerization domain.

Proteolytic cleavage site was shown (AA 104). The C-terminal globular domain makes up the majority of the protein.

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Chapter 2

General Experimental Methods and Materials

2.1. Experimental Animals

1-day, 4-day, 7-day, 10-day, 14-day, and 6-week old New Zealand White rabbits of either sex were used in these studies. Animals were cared for according to the guidelines outlined by the Canadian Council for Animal Care.

The plasma samples from 6-week-old rabbits were collected from the animals that have been utilized for other experimental procedures by Mrs. Donna Panas (Dr. Ivan Rebeyka's Laboratory).

2.1.1. Measurement of Heart Carnitine Content

Samples of frozen tissue were powdered in liquid N_2, and then homogenized in 3% perchloric acid and after centrifugation (800 x g) at 4°C for 5 min the supernatant was neutralized by KOH. Free carnitine, short chain carnitine, and long chain acylcarnitine was extracted from tissue as previously described (Idell-Wenger et al 1978, McGarry and Foster 1985). For the measurement of *free carnitine*, a part of the extract was assayed without further treatment. Carnitine esters were hydrolyzed by mixing one part of the remaining extract with 11 M KOH and incubating at 37°C for one hour. Thereafter, the samples were neutralized and assayed for *short-chain acylcarnitines*. *Long-chain acylcarnitines* are insoluble in acid and precipitate with the denatured tissue protein. Therefore, the amount of long-chain acylcarnitines was determined as free carnitine after treatment of the acid-insoluble fraction of the tissue with alkali and subsequent neutralization.

The reaction mixture contained in a volume of 1.2 ml, 100 mM Hepes buffer, pH 7.4, 2 μmol sodium tetrathionate, 25 μM acetyl CoA (including [^{3}H]acetyl CoA), 2 mM sodium tetrathionate and 1 U carnitine acetyltransferase, and 200 μl of the extract. L-carnitine standards were assayed in equal conditions as the samples. The reactions were initiated by addition of the sample to the test tube that contained other components of the reaction mixture. After standing at room temperature for 30 min, Dowex 1-X10, 200-400 mesh anion exchange resin was added to remove excess [^{3}H]acetyl CoA. The amount of [^{3}H]acetylcarnitine was counted using standard scintillation counting procedures. The carnitine content of the sample is obtained by reference to the standard curve.

2.1.2. CPT I Isoform Expression

The expression of M-CPT I and L-CPT I was measured by RT-PCR of total RNA isolated from neonatal frozen rabbit heart tissue. 1 µg of RNA was used in the reactions. The PCR ELISA kit (Roche Diagnostics) was used for this analysis. This system allows for non-radioactive detection of PCR products in a microplate. It is based on the incorporation of DIG-dUTP (digoxygenin labeled dUTP) during PCR. These labeled PCR products were then bound to the streptavidin-coated surface of a microplate by using biotin–labeled capture probes. The capture probes were designed to hybridize to the internal sequences of the PCR products. The DIG labelled PCR products were detected with an anti-DIG-alkaline phosphatase conjugate and AttoPhos substrate using a fluorescence microplate reader. The primer sequences and biotin capture probe sequences used in these experiments were: L-CPT I, P1 5'–GTGAAGAAACAACCCCCAGA-3', P2 5'-GGAAGCACTTGAGA CAAGCC-3', biotin-capture probe 5'-ATCACCTTGTTTGGCCTCAC-3'; M-CPT I, P1 5'-TGTCATGGCAACAGTTGGTT-3', P2 5'-TTGTCCTGGAATTCTTTGGC-3', biotin capture probe 5'-CCGACAAGGTATGGCTCCTA-3'. The primers were designed from the published sequences for rat M-CPT I and L-CPT I. DNA and protein homology searches were performed with BLAST, using GenEMBL or SWISS-PROT databases.

2.1.3. Preparation of Unperfused Heart Tissues

Fetal rabbits were used only for the measurement of myocardial carnitine content. The pregnant doe (29-day gestation) was anaesthetized with sodium pentobarbital (Euthanyl® 60 mg/kg) via the lateral ear vein. Following anesthesia, fetal rabbits were removed from the doe's uterus and each rabbit was injected with sodium pentobarbital (60 mg/kg i.p.). The throracic cage was then opened and the hearts were quickly excised. The hearts were rinsed briefly in ice-cold Krebs-Henseleit solution (25 mM $NaHCO_3$, 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 11mM glucose, and 2.5 mM $CaCl_2$) and frozen using Wollenberger tongs pre-cooled to the temperature of liquid nitrogen. All hearts were stored at -80°C until used for analysis.

For the preparation of hearts from 1-day, 4-day, 7-day, 10-day, and 2-week old rabbits, the animals were injected with sodium pentobarbital (60 mg/kg i.p.). The throracic cage was opened and hearts were excised. The hearts were rinsed briefly in ice-cold Krebs-Henseleit solution and frozen using Wollenberger tongs pre-cooled to the temperature of liquid nitrogen. All hearts were stored at -80°C until used for biochemical analysis.

2.2 Heart Perfusions

2.2.1. Langendorff Perfusions of 1-day-old Rabbit Hearts

Rabbits were anesthetized with an intraperinoteal injection of 60 mg/kg pentobarbital, and the thoracic cavity was opened. The entire heart, lungs, and immediate vasculature were quickly excised and immersed in ice-cold Krebs-Henseleit solution. The aorta was cannulated and a retrograde perfusion at constant pressure of 50 mm Hg through the aorta was initiated with Krebs-Henseleit a 37°C solution (pH 7.4), gassed with 95% O_2-5% CO_2 (Buffer 1). The lungs were removed and the system was sealed for the measurement of metabolic parameters. As soon as the system was sealed, the line that carries Buffer 1 was clamped. At the same time, the recirculating line that carries the modified Krebs-Henseleit buffer was opened. This buffer contained 0.4 mM [1-^{14}C]palmitate prebound to 3% bovine serum albumin in addition to the other components of the Krebs-Henseleit solution (Buffer 2). Hearts were perfused for a 40 min aerobic perfusion period during which palmitate oxidation rates were determined (described below). In specific protocols, hormones were added to Buffer 2 as outlined in the "Experimental Procedures" sections of individual chapters.

At the end of the perfusion protocols, hearts were quickly frozen with Wollenberger tongs pre-cooled to the temperature of liquid nitrogen.

2.2.2. Isolated Working Heart Perfusions

Hearts from 7-day-old rabbits were perfused using the working heart model (Neely et al 1967). Once the hearts were excised from the animal and cannulated via the aorta, Buffer 1 was delivered via the aortic cannula, initiating a Langendorff perfusion. Lungs as well as other excess tissue and fat were trimmed. The hearts were then switched to working mode and subjected to a preload pressure of 7.5 mm Hg and an aortic afterload of 30 mm Hg as previously described by Itoi and Lopaschuk (1996). Isolated working hearts were perfused with modified Krebs-Henseleit solution that contains radioactive-labeled glucose and palmitate (Buffer 2,3, or 4). In some experiments, hormones were included as outlined in the "Experimental pocedures" sections of individual chapters. Heart rate and peak systolic pressure were measured by a pressure transducer (Harvard Apparatus) installed in the aortic outflow line. Data were collected using an MP100 system from AcqKnowledge (BIOPAC Systems, Inc.). Cardiac output and aortic flows were obtained by monitoring the flows into the left atria and from the afterload line using Transonic flow probes, respectively. Cardiac work was calculated as peak systolic pressure x cardiac output x 10^{-3}.

Both Langendorff perfusions of 1-day-old hearts and working heart perfusions of 7-day-old hearts were performed using spontaneously beating hearts.

2.3. Measurement of Metabolic Parameters

2.3.1. Palmitate Oxidation Rates in 1-day-old Rabbit Hearts

Palmitate oxidation rates were determined by the quantitative collection of $^{14}CO_2$ produced (from Krebs' Cycle activity) during the aerobic perfusion of the heart with the Buffer 2 containing [1-^{14}C]palmitate, as described previously

(Lopaschuk et al 1994, Lopaschuk and Barr 1997) (Figure 2.2). Hearts were perfused in a closed recirculating system using an oxygenator with a large surface area in constant contact with 95% O_2 and 5% CO_2. This ensured that the perfusate was adequately oxygenated. Perfusate and exhaust gas samples from the sealed system were collected at 10-, 20-, 30-, and 40 min time points during the 40 min perfusion protocol. The exhaust gas was trapped by bubbling it through 25 or 40 ml of 1 M benzethonium hyamine hydroxide. Buffer samples (2 ml) and duplicates of benzethonium hyamine hydroxide (0.3 ml x 2) sample were removed at each time period.

To extract $^{14}CO_2$ trapped in the buffer samples, two 1 ml buffer samples were each injected into a test tube containing 0.5 ml 9 N H_2SO_4. Contact with this acid liberated $^{14}CO_2$ from the ^{14}C-labelled bicarbonate. $^{14}CO_2$ was trapped by the benzethonium hyamine hydroxide-soaked filter paper that was placed in the scintillation vial secured with a rubber stopper to the top edge of the test tube. The test tubes were vortexed for 10 seconds and left at room temperature for at least 1 hour. Both the vials (containing the benzethonium hyamine hydroxide-soaked filter paper) and gaseous $^{14}CO_2$ trapped in the 0.3 ml of the benzethonium hyamine hydroxide were counted using standard scintillation counting procedures (Figure 2.3). Palmitate oxidation rates were expressed as nmol palmitate/ min/ total dry weight of the heart.

2.3.2. Glucose Oxidation, Palmitate Oxidation, and Glycolysis in 7-day-old Rabbit Hearts

Glucose oxidation and palmitate oxidation rates were measured simultaneously by perfusing the hearts with both [U-^{14}C]glucose and [9,10-^{3}H]palmitate (Buffer 3) (Barr and Lopaschuk 1997). Samples were collected by 10 min intervals. Glucose oxidation rates were determined as described above by trapping and measuring $^{14}CO_2$ released by the metabolism of [U-^{14}C]glucose.

Palmitate oxidation was determined by measuring $^{3}H_2O$ produced from [9,10-^{3}H]palmitate. To separate the $^{3}H_2O$ in the perfusate from [9,10-^{3}H]palmitate, 200 μl samples from the perfusate were placed in capless microcentrifuge tubes. These tubes were then carefully placed in 3 ml plastic vials that had 500 μl water in them. The vials were capped and incubated at 50°C for 24 hours. Only ^3H_2O water from the microcentrifuge tube was transferred to the water in the vial. To determine the efficiency of the transfer, a separate perfusate solution was prepared without the addition of radioactive palmitate or glucose. ^3H_2O was added to this buffer and treated the same way as the buffer samples from the perfusate. At the end of the transfer, microcentrifuge tubes were removed and counted using standard scintillation counting procedures.

To measure glycolysis, [5-^{3}H]glucose was added to the perfusate in a separate series of perfusions (Buffer 4). ^3H_2O released from the metabolism of [5-^{3}H]glucose was separated from [^{3}H]glucose as described previously (Rovetto et al 1975). Briefly, Dowex 1-X4 anion exchange resin (200-400 mesh) was suspended in 0.2 M potassium tetraborate, and placed in plastic columns. The Dowex in the columns was extensively washed with H_2O before use. A 0.2 ml of the buffer sample (in duplicate) was run through the column and eluted into scintillation vials with 0.8 ml H_2O. Samples were subjected to standard scintillation counting procedures.

At the end of the perfusions, hearts were quickly clamped with tongs that had been cooled to the temperature of liquid nitrogen. Frozen ventricular tissue was weighed, powdered (using mortar and pestle that had been cooled to the temperature of liquid nitrogen), and stored in cryovials at -80°C until use. A small portion of the powdered ventricular tissue was used to determine the dry to wet ratio of the ventricles. This ratio was used to determine the total dry weight of the heart from the known wet weights of the atrial and ventricular tissues.

2.4. Tissue Preparation for Enzyme Assays

2.4.1. Preparation of Mitochondrial Fraction from Fresh Heart Tissues

Mitochondria were prepared by differential centrifugation using a modified method of Power et al (1994). Briefly, hearts from 1-day, 7-day, and 10-day-old rabbit hearts were excised and finely minced in with scissors in 10 ml of homogenization buffer (0.25 M sucrose, 5 mM Tris-HCl and 1 mM EGTA, pH 7.4). Two hearts were pooled in the preparation of the mitochondria from 1-day-old hearts due to the low yield. The crude homogenate was centrifuged at 800 x g for 10 min at 4°C. The resulting pellet was washed by resuspension in 2 vol. of homogenization buffer and was then re-centrifuged at 800 x g. This step was repeated twice to maximize the yield of mitochondria. The combined supernatants were centrifuged at 6000 x g for 15 min, and the resultant pellet was carefully resuspended in 2 ml of homogenization buffer, and centrifuged at 6000 x g for 15 min. The resultant pellet (crude mitochondria) was gently resuspended in 2 ml of homogenization buffer. The crude mitochondrial fraction (0.5 ml) was layered onto 9 ml of 30% Percoll and centrifuged at 50,000 x g for 60 min at 4 °C. The bottom mitochondrial protein band was collected.

2.4.2 Tissue Homogenization for AMPK and ACC Activity Assays

Approximately 50 mg frozen powdered ventricular tissue from previously perfused hearts were homogenized using a Polytron® homogenizer for 30 seconds at 4°C in 400 μl buffer containing 50 mM Tris-HCl (pH 7.5), 0.25 M mannitol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM sodium flouride, 5 mM sodium pyrophosphate, 10% (w/v) glycerol, and 0.1% (v/v) of mammalian protease inhibitor cocktail (Sigma). Following centrifugation at 9000 x g for 5 min at 4°C, protein content was measured using Bradford's method (Bradford 1976). For the AMPK activity measurements, 0.1% (v/v) Triton X-100 was included in the protein samples.

2.4.3. Tissue Homogenization for MCD Assay

Frozen ventricular tissue (15-20 mg) from previously perfused hearts were homogenized using a Polytron® homogenizer for 30 seconds at 4°C in a buffer consisting of 75 mM KCL, 20 mM sucrose, 10 mM HEPES, 1 mM EGTA, 50 mM NaF, and 5mM NaPPi (Dyck et al 1998). Following centrifugation at 9000 x g for 5 min at 4°C, protein content was measured using Bradford's method (Bradford 1976).

2.5. Enzyme Assays

2.5.1. CPT I Activity Assay

CPT I activity was assayed in the purified mitochondrial fraction (described in Section 2.4.1) of rabbit hearts in the direction of acylcarnitine formation using palmitoyl CoA and carnitine as substrates (McGarry and Brown 2000). Reactions were carried out at 30°C in glass culture tubes (13mm x 100mm). Final concentrations in a total of 500 µl were: 75 µM palmitoyl CoA, 4 mM ATP, 4 mM $MgCl_2$, 0.25 mM glutathione, 40 µg/ml rotenone, 2 mM KCN, 15 mM KCl, 1% (w/v) BSA and 105 mM Tris-HCl, pH 7.4. The amount of the mitochondrial protein ranged between 35 and 40 µg in a total volume of 50 µl. Palmitoyl CoA was dissolved in 25 mM KH_2PO_4, pH 5.3. The mitochondria were initially incubated with the assay buffer and palmitoyl CoA for 3 min at 30°C. Reactions were initiated by the addition of L-[^{14}C]carnitine to a final L-carnitine concentration of 10-1500 µM and the incubation continued for a further 6 min. Reactions were stopped by adding 100 µl 11 M HCl. The [^{14}C]palmitoylcarnitine formed was extracted using 1-butanol (Bremer and Norum 1967). The incubation mixture (500 µl + 100 µl HCl) was diluted to 2 ml with butanol-saturated water, and [^{14}C]palmitoylcarnitine was extracted with 1 ml of water-saturated butanol. The mixture was vortexed and centrifuged at 3500 rpm for 7 min. The butanol phase was removed and washed twice with 2x of its volume with butanol-saturated water. Finally, the radioactivity in 500 μl of butanol extract was measured using standard scintillation techniques, and the content of palmitoylcarnitine was calculated from the known specific activity of carnitine used.

2.5.2. AMPK Activity Assay

AMPK activity was assayed in whole tissue homogenates by following the incorporation of [γ-^{32}P]ATP into the synthetic peptide AMARA (Kudo et al 1996). The assay was performed in a 25 μl total volume containing 40 mM HEPES-NaOH (pH 7.0), 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 200 μM AMARA peptide, 0.8 mM dithiothreitol, 5 mM $MgCl_2$, 200 μM ATP (containing [γ-^{32}P]ATP). The assay was performed in the absence or presence of 200 μM 5'-AMP at 30°C for 5 min. The reaction was initiated by the addition of 200 μM ATP and 5 mM $MgCl_2$. At the end of reaction, 15 μM aliquots were removed and spotted on a 1 x 1 cm square of phosphocellulose paper (Whatman®, p81), which were then placed into 150 mM phosphoric acid. These papers were washed 4 times for 10 min with 150 mM phosphoric acid and then once with acetone. The radioactivity of the dried papers was determined using standard liquid scintillation procedures.

2.5.3. ACC Activity Assay

ACC activity was measured using the [^{14}C]CO_2 fixation method (Kudo et al 1995). The assay mixture contained 60.6 mM Tris-acetate (pH 7.5), 1 mg/ml bovine serum albumin, 1.3 μM 2-mercaptoethanol, 2.1 mM ATP, 1.1 mM acetyl CoA, 5 mM Mg-acetate, 18.2 mM $NaHCO_3$ (containing $NaH^{14}CO_3$). Following a 2 min incubation at 37°C, in the absence and presence of 10 mM citrate, the reaction was stopped by adding 25 μl of 10% perchloric acid. Following centrifugation at 2000 x g for 20 min, radioactivity of the supernatant was determined using standard liquid scintillation counting procedures.

2.5.4. MCD Activity Assay

MCD activity was measured using a radiometric assay as described previously by Dyck et al (1998). Briefly, heart homogenates were incubated in an incubation buffer containing 0.1 M Tris, pH 8, 0.5 mM dithiothreitol, 50 mM NaF, 5 mM NaPPi, and 1 mM malonyl CoA for 10 min at 37°C. The reaction was stopped by the addition of 40 μl perchloric acid (0.5 mM), neutralized with 10 μl of 2.2 M $KHCO_3$ (pH 10) and centrifuged at 10,000 x g for 5 min to remove the precipitated proteins. The incubation of the heart sample with malonyl CoA allowed for the conversion of malonyl CoA to acetyl CoA, which was then combined with [^{14}C]oxaloacetate to produce [^{14}C]citrate. Unreacted [^{14}C]oxaloacetate was removed from the reaction mixture by the addition of 6.8 mM sodium glutamate and 0.533 μU/μl aspartate aminotransferase, followed by a 20 min incubation at room temperature. The resulting solution was then stirred in a 1:2 suspension of Dowex AG 50W-8X resin (100-200 mesh) and centrifuged at 400 x g for 10 min. The supernatant was counted for ^{14}C present in the form of [^{14}C]citrate. The amount of acetyl CoA produced by MCD was quantified by using an acetyl CoA concentration standard curve that had been subjected to the identical assay conditions.

2.6. Analysis of Enzyme Expression

2.6.1. Electrophoresis and Immunoblotting

To determine the expression of AMPK, ACC, and MCD enzymes, homogenates that have been prepared for enzyme activity assays were subjected to sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970).

Following gel electrophoresis, the fractionated proteins were transferred to nitrocellulose membrane using a wet transfer method. Transfer buffer contained 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. Membranes were then blocked with 10% (w/v) skim milk powder in Tris-buffered saline with 0.05%

Tween 20 for 1 hour at room temperature. For immunoblotting, membranes were incubated with an appropriate amount of mono or polyclonal antibody against the protein of interest in 1% (w/v) skim milk powder in Tris-buffered saline with 0.05% Tween 20 at 4°C overnight. Membranes were washed 3 times with Tris-buffered saline with 0.05% Tween 20. Membranes were probed with a 1:1000 dilution of horseradish peroxidase (HRP)-conjugated secondary antibody in 1% (w/v) skim milk powder in Tris-buffered saline with 0.05% Tween 20. Membranes were washed 3 times with Tris-buffered saline with 0.05% Tween 20. Proteins were visualized using an ECL Western blotting kit.

For ACC enzyme expression, membranes were incubated in a 1:500 dilution of HRP-conjugated streptavidine in 1% (w/v) skim milk powder in Tris-buffered saline with 0.05% Tween 20. Membranes were washed 3 times with Tris-buffered saline with 0.05% Tween 20. Proteins were visualized using an ECL Western blotting kit.

Sources and dilutions of the primary antibodies are indicated in the "Experimental Procedures" sections of individual chapters.

Quantitative Western blotting technique of plasma adiponectin is described in detail in the "Experimental Procedures" section of Chapter 5.

2.7. Determination of CoA Esters

CoA esters were determined on 6% perchloric acid (PCA) extracts from frozen heart tissues, as described previously (Lopaschuk et al 1994). Briefly, frozen ventricular tissues from previously perfused hearts were homogenized using a Polytron® homogenizer in 6% PCA containing 1 mM DTT. Homogenates were centrifuged at 10,000 x g for 10 min. The CoA esters in the supernatant were measured using a modified HPLC method originally described by King et al (1988).

Extraction of CoA esters was performed by myself. HPLC protocols were carried out by Mr. Kenneth Strynadka.

2.8. Recombinant Adiponectin Production and Characterization

Recombinant adiponectin was produced by following the protocol described in Fruebis et al (2001) except that cDNA was cloned in pET-30a (Novagen), and a Chelating Sepharose Fast Flow column was used to isolate the N-terminal His_6-tagged fusion protein from the lysed bacterial pellet.

The globular region of adiponectin was prepared from the full-length form by enzymatic cleavage (Fruebis et al 2001). Briefly, purified adiponectin was incubated with acetylated trypsin-type V-S from bovine pancreas in PBS at 400 units/mg protein at 25°C for 10 min. The reaction was stopped by running the sample through a PolyPrep Column containing immobilized trypsin inhibitor (Pierce) at 4°C. Fractions of proteins were then concentrated on an Amicon Centricon Filter (Millipore) with a molecular weight cutoff of 10,000 and stored at -80°C until use. To detect both full-length adiponectin and gAd, the end products were separated by SDS-PAGE, and transferred onto nitrocellulose paper by standard procedures. HRP-conjugated anti-His-tag antibody (Santa Cruz) and rabbit anti-adiponectin antibody raised against the globular head domain (Chemicon) followed by HRP-conjugated anti-rabbit IgG (Santa Cruz) was used to visualize the proteins.

Cloning of the cDNA in pET-30a and optimization of the FPLC procedure were carried out by Ms. Judith Altarejos.

2.9. Statistical Analysis

Data are expressed as mean ± S.E. The unpaired Students t-test was used to determine statistical significance between two separate group means. One-way ANOVA followed by Tukey-Kramer was used when two subsequent measurements in one group were compared with two subsequent measurements in the other. A value of $p<0.05$ was regarded as significant.

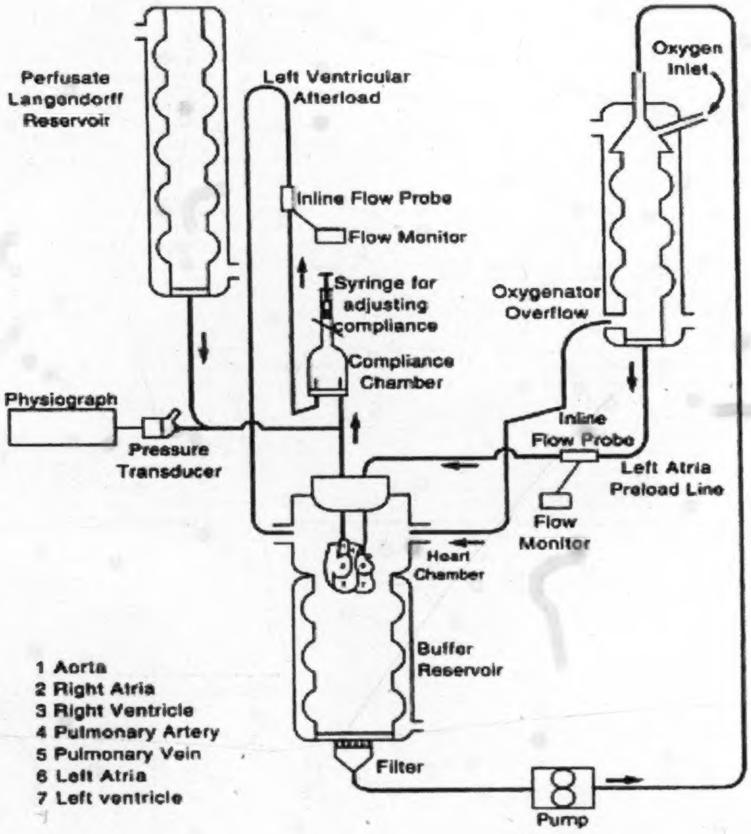

Perfusate Langendorff Reservoir
Left Ventricular Afterload
Oxygen Inlet
Inline Flow Probe
Flow Monitor
Syringe for adjusting compliance
Oxygenator Overflow
Compliance Chamber
Physiograph
Pressure Transducer
Inline Flow Probe
Left Atria Preload Line
Flow Monitor
Heart Chamber
Buffer Reservoir
1 Aorta
2 Right Atria
3 Right Ventricle
4 Pulmonary Artery
5 Pulmonary Vein
6 Left Atria
7 Left ventricle
Filter
Pump

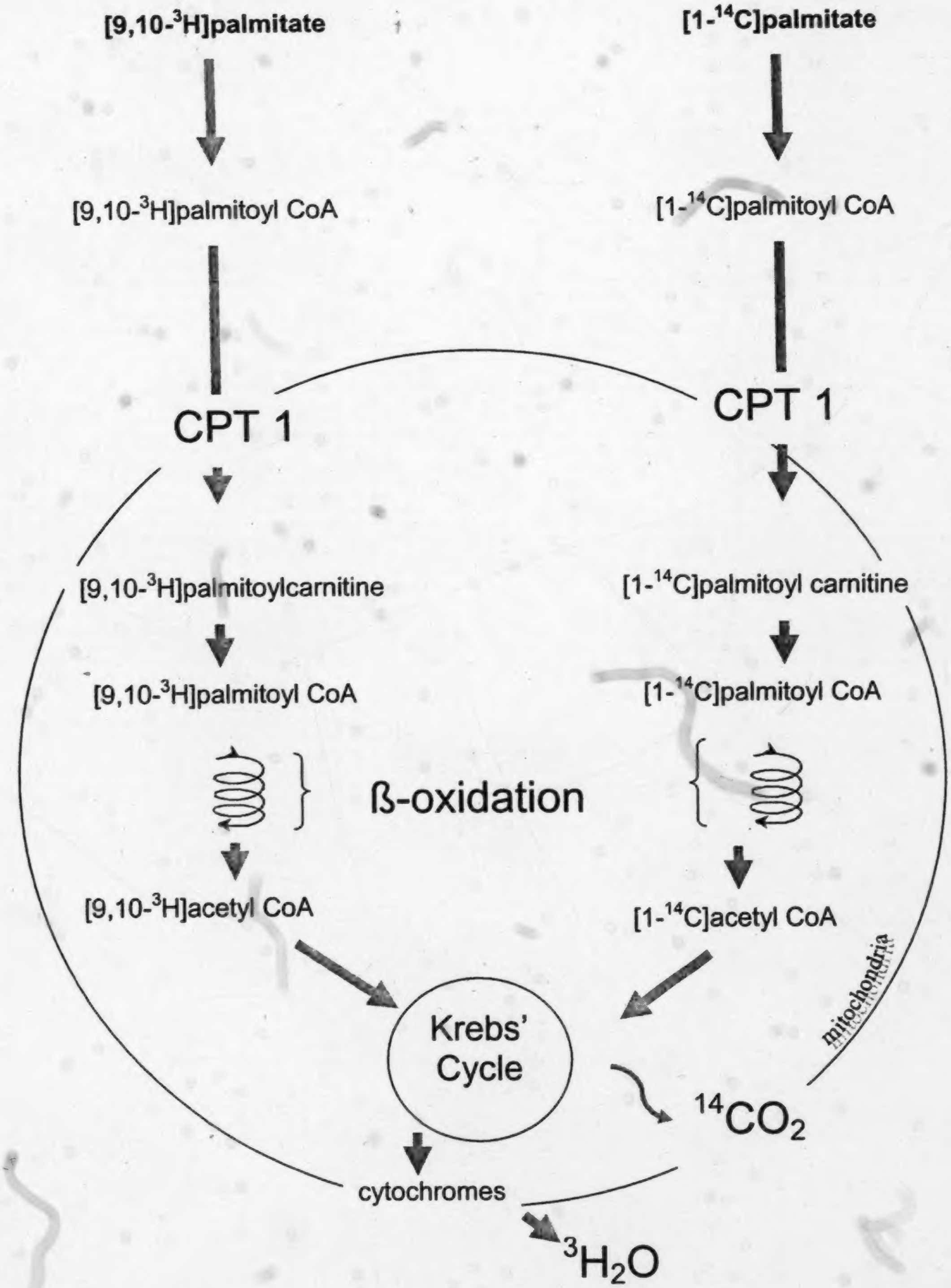
Figure 2.1
Diagram of isolated working heart perfusion apparatus

The recirculating buffer flows through the system starting from the buffer reservoir. It is pumped from the reservoir to the top of the oxygenator where it forms a thin film over the surface of the oxygenator and picks up O_2 and CO_2. Perfusate is delivered to the left ventricle via a cannula connected to the left atria. Any buffer that does not enter the heart goes through the overflow on the oxygenator. The perfusate is then ejected from the left ventricle through the cannulated aorta. The buffer spills over the top of the afterload line and falls freely down to the heart chamber where it returns to the buffer reservoir.

Figure 2.2. Measurement of palmitate oxidation using radio-labeled fatty acids

When [9,10-^{3}H]palmitate is used, ^3H_2O is separated from the unused [9,10-^{3}H]palmitate as explained in detail in Section 2.3.2 of this chapter.

When [1-^{14}C]palmitate is used, $^{14}CO_2$ that is produced by the heart is collected as explained in Section 2.3.2 of this chapter and illustrated in Figure 2.3.


[9,10-^{3}H]palmitate
[1-^{14}C]palmitate
[9,10-^{3}H]palmitoyl CoA
[1-^{14}C]palmitoyl CoA
CPT 1
CPT 1
[9,10-^{3}H]palmitoylcarnitine
[1-^{14}C]palmitoyl carnitine
[9,10-^{3}H]palmitoyl CoA
[1-^{14}C]palmitoyl CoA
ß-oxidation
[9,10-^{3}H]acetyl CoA
[1-^{14}C]acetyl CoA
mitochondria
Krebs' Cycle
$^{14}CO_2$
cytochromes
$^{3}H_2O$

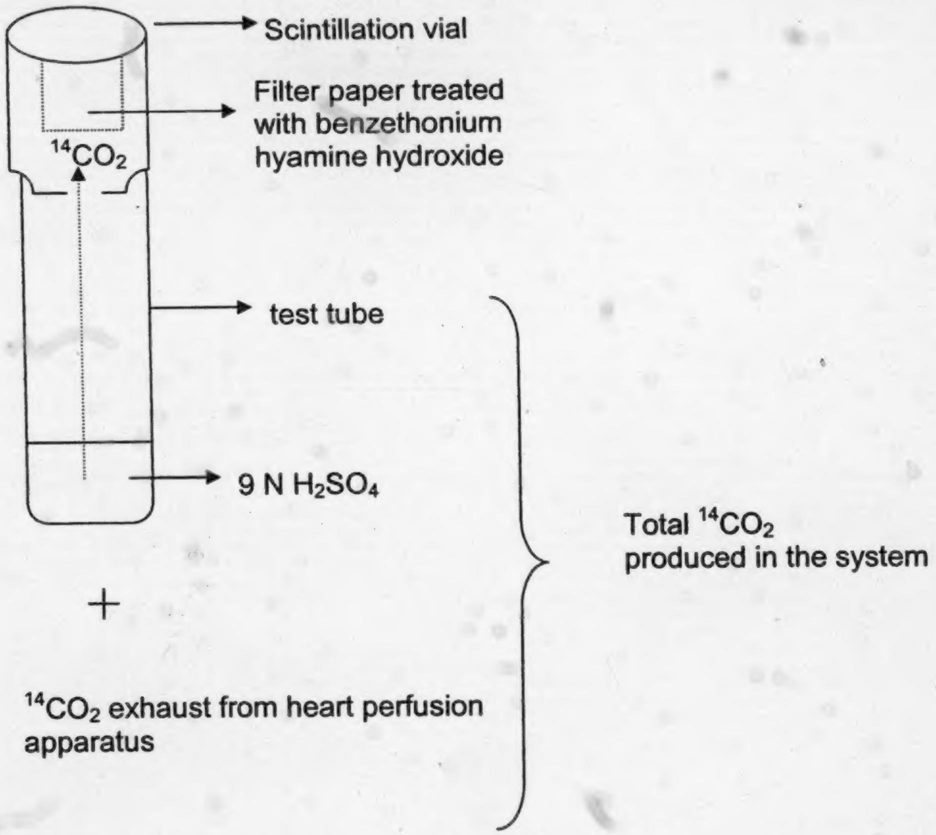

Scintillation vial
Filter paper treated with benzethonium hyamine hydroxide
$^{14}CO_2$
test tube
9 N H_2SO_4
+
$^{14}CO_2$ exhaust from heart perfusion apparatus
Total $^{14}CO_2$ produced in the system

Figure 2.3. Collection of $^{14}CO_2$

$^{14}CO_2$ that is released by the heart is released into the air or trapped in the buffer as bicarbonate. By using a sealed experimental system, all the CO_2 that is normally released into the air and exits the perfusion apparatus is trapped using benzethonium hyamine hydroxide. $^{14}CO_2$ that is trapped in the buffer as bicarbonate is released by adding aliquots of buffer sample to sealed test tubes containing H_2SO_4. This CO_2 is then trapped by a piece of filter paper that is treated with benzethonium hyamine hydroxide and placed inside the scintillation vial as shown in the figure. Radioactivity on the filter paper and timed samples taken from the benzethonium hyamine hydroxide trap were added together to calculate the total $^{14}CO_2$ production in the system.

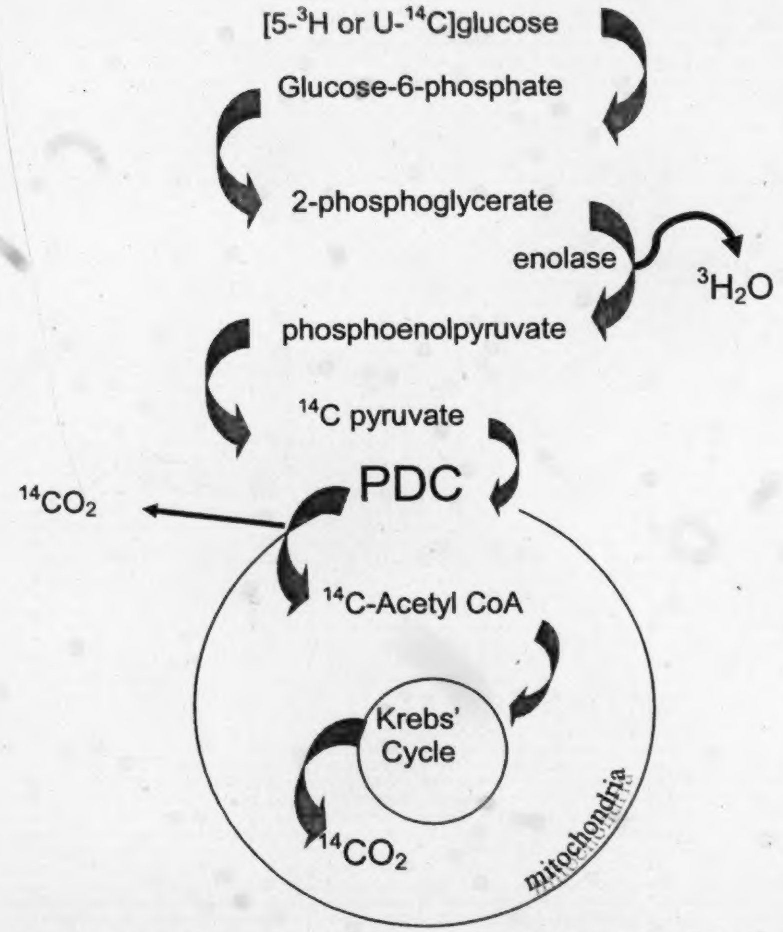

[5-3H or U-14C]glucose
Glucose-6-phosphate
2-phosphoglycerate
enolase
3H2O
phosphoenolpyruvate
14C pyruvate
PDC
14CO2
14C-Acetyl CoA
Krebs' Cycle
14CO2
mitochondria

Figure 2.4. Measurement of glycolysis and glucose oxidation using radio-labeled glucose

To measure glycolysis, [5-^{3}H]glucose is added to the perfusate. At the enolase step of glycolysis, ^{3}H is released as ^3H_2O. This ^3H_2O is separated from the unused [5-^{3}H]glucose as explained in detail in Section 2.3.2 of this chapter.

During the oxidation of glucose, the heart releases CO_2 when acetyl CoA is decarboxylated by PDC and when acetyl CoA passes through the Krebs' Cycle. This $^{14}CO_2$ is collected as explained in Section 2.3.2 of this chapter and illustrated in Figure 2.3.

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Chapter 3

The Relative Importance of Malonyl CoA and Carnitine in the Maturation of Fatty Acid Oxidation in the Newborn Rabbit Heart

A version of this chapter was published in American Journal of Physiology-Heart and Circulatory Physiology:

Besikci, A.O., Campbell, F.M., Hopkins, T.A., Dyck, J.R., Lopaschuk, G.D. *Relative importance of malonyl CoA and carnitine in maturation of fatty acid oxidation in newborn rabbit heart*. Am. J. Physiol. 284: H283-H289, **2003**.

CPT I isoform expression experiments were performed cooperatively between Dr. Fiona M. Campbell and myself.

PDH activity assays were performed by Ms. Teresa Hopkins

MCD activity assays were performed by Mr. Rick Barr

3.1. Introduction

Fatty acid oxidation in the newborn heart matures rapidly following birth (Werner et al 1989, Lopaschuk and Spafford 1990). For example, in one-day old rabbit hearts, fatty acid oxidation rates are very low, and contribute less than 10% to overall ATP production (Lopaschuk and Spafford 1990). However, by 7-days of age fatty acid oxidation dramatically increases, and becomes the predominant source of energy production. What is responsible for this increase in fatty acid oxidation has not been completely delineated, although an increase in mitochondrial uptake of fatty acids has been implicated in this process (Girard 1992, Lopaschuk et al 1994a, Brown et al 1995).

The transport of fatty acids into the mitochondria is primarily controlled at the level of carnitine palmitoyltransferase I (CPT I) (McGarry and Foster 1980). CPT I is in turn inhibited by malonyl CoA and stimulated by L-carnitine (McGarry and Foster 1980; McGarry et al 1983; Weis et al 1994a; Weis 1994b, Brown et al 1995). The heart expresses two isoforms of CPT I, L-CPT I and M-CPT I, with L-CPT I being less sensitive to inhibition by malonyl CoA, and requiring lower L-carnitine concentrations for maximal stimulation (McGarry and Foster, 1980, McGarry et al 1983; Weis et al 1994a; Weis et al 1994).

The dramatic increase in fatty acid oxidation seen in the newborn heart has been attributed to an increase in L-carnitine levels and a switch from the L-CPT I isoform to the M-CPT I isoform (Brown et al 1995). However, since M-CPT I is more sensitive to inhibition by malonyl CoA this switch should actually lead to a decrease in fatty acid oxidation rates, not an increase as previously observed (Werner et al 1989, Lopaschuk and Spafford 1990, Lopaschuk et al 1994b). An alternate explanation for the increase in fatty acid oxidation is a decrease in myocardial malonyl CoA levels following birth, resulting in a decrease in malonyl CoA inhibition of CPT I. Indeed, we have previously shown that following birth, malonyl CoA levels do dramatically decrease in the newborn heart. In one-day old rabbit hearts, malonyl CoA levels are very high, but decrease dramatically by

7-days following birth (Lopaschuk et al 1994b, Dyck et al 1998, Makinde et al 1997). These changes in malonyl CoA can be explained by alterations in the control of both malonyl CoA synthesis and degradation (Lopaschuk et al 1994a, Lopaschuk et al 1994b, Dyck et al 1998; Makinde et al 1997).

Malonyl CoA is synthesized in the heart by acetyl-CoA carboxylase (ACC), the activity of which decreases in the heart following birth (Lopaschuk et al 1994). ACC is in turn phosphorylated and inactivated by 5'-AMP-activated protein kinase (AMPK) (Hardie 1992), the activity and expression of which increases in the heart following birth (Makinde et al 1997). Malonyl CoA is degraded in the heart by decarboxylation to acetyl CoA by malonyl CoA decarboxylase (MCD) (Lopaschuk et al 1994). The relative importance of changes in MCD and ACC control of malonyl CoA versus changes in L-carnitine control of CPT I or alterations in CPT I activity/expression, to the increase in fatty acid oxidation in the newborn heart is not clear.

In addition to fatty acid oxidation, glucose oxidation is an important source of energy for the adult heart. In contrast to fatty acid oxidation following birth, rates of glucose oxidation are low in the newborn heart (Lopaschuk and Spafford 1990) and do not fully mature until after weaning (Werner et al 1989). Pyruvate decarboxylation is the key rate-limiting irreversible step in glucose oxidation and is catalyzed by the pyruvate dehydrogenase multienzyme complex (PDH). Activity of PDH is controlled by kinase-mediated inactivation, responsive to acetyl CoA/CoA and NADH/NAD$^+$ ratios and a phosphatase-mediated activation, which responds to mitochondrial calcium and magnesium concentrations. In the adult heart, fatty acids are potent inhibitors of glucose oxidation in the heart, secondary to an increase in the acetyl CoA/CoA and NADH/NAD$^+$ ratio that result from the oxidation of fatty acids (Patel and Roche 1990). Whether glucose oxidation remains low in the newborn heart due to a delay in the maturation of the enzymes involved in glucose oxidation, or to the increase in fatty acid oxidation is not known.

The first aim of this study was to determine the relative contributions of CPT I isoform expression, myocardial L-carnitine content and the kinetics of CPT I to the maturation of fatty acid oxidation in the newborn heart. The second aim of this study was to determine if the low glucose oxidation rates seen in the newborn period are due to a low PDH activity and/or a low capacity for glucose oxidation.

3.2. Experimental Procedures

Heart Tissue Isolation

Hearts from 1-day, 3-day-, 7-day, 10-day, and 14-day-old rabbits from either sex were used in this study. Hearts from anesthetized rabbits were removed and placed in ice-cold Krebs Henseleit solution as described in Chapter 2. Hearts were then used either for the preparation of mitochondria, cannulated for isolated heart perfusions, or frozen immediately with tongs cooled in liquid N_2. Metabolic rates were calculated using the total dry mass of the heart to correct for variations in heart size.

Measurement of Heart Carnitine Content

Samples of frozen tissue were powdered in liquid N_2, and then homogenized in 3% perchloric acid and after centrifugation the supernatant was neutralized by KOH. Free carnitine, short chain carnitine, and long chain acylcarnitine was extracted from tissue as previously described (Idell-Wenger et al 1978). Carnitine levels were measured using a radioisotopic assay described in Section 2.1.1.

CPT I Isoform Expression

The expression of M-CPT I and L-CPT I was measured by RT-PCR of total RNA isolated from neonatal frozen rabbit heart tissue. 1 μg of RNA was used in the reactions. The PCR ELISA kit (Roche Diagnostics) was used for this analysis. This system allows for non-radioactive detection of PCR products in a microplate. It is based on the incorporation of DIG-dUTP (digoxygenin labeled dUTP) during PCR. These labeled PCR products were then bound to the streptavidin-coated surface of a microplate by using biotin–labeled capture probes. The capture probes were designed to hybridize to the internal sequences of the PCR products. The DIG labelled PCR products were detected with an anti-DIG-alkaline phosphatase conjugate and AttoPhos substrate using a fluorescence microplate reader. The primer sequences and biotin capture probe sequences used in these experiments were: L-CPT I, P1 5'–GTGAAGAAACAACCCCCAGA-3', P2 5'-GGAAGCACTTGAGA CAAGCC-3', biotin-capture probe 5'-ATCACCTTGTTTGGCCTCAC-3'; M-CPT I, P1 5'-TGTCATGGCAACAGTTGGTT-3', P2 5'-TTGTCCTGGAATTCTTTGGC-3', biotin capture probe 5'-CCGACAAGGTATGGCTCCTA-3'. The primers were designed from the published sequences for rat M-CPT I and L-CPT I. DNA and protein homology searches were performed with BLAST, using GenEMBL or SWISS-PROT databases.

Preparation of Mitochondria

Mitochondria were prepared by differential centrifugation using a modified method of Power et al (Power et al 1994) as described in Section 2.4.1.

CPT I Activity Assay

CPT I activity was assayed in the direction of acylcarnitine formation using palmitoyl CoA and carnitine as substrates (Esser et al 1993) as described in Section 2.5.1. Before detailed kinetic analysis of the enzyme activity, time- and protein-dependence of the assay was confirmed as shown in the "Results" section of this chapter.

PDH Activity Assay

PDH activity (both activate and total activity) was measured using a revised protocol (Collins-Nakai 1994, Constantin-Teodosiu 1991) based on the radiometric assay described by Constantin-Teodosiu et al (1991).

MCD Activity Assay

MCD activity was measured as described in Section 2.5.4.

3. 3. Results

3.3.1. L-Carnitine Content in Newborn Hearts

The amount of free carnitine, long chain acyl carnitine and short chain acyl carnitine in 1-day-old, 4-day-old, 10-day-old and 14–day-old rabbit is shown in Table 3.1. Myocardial free carnitine content did not vary dramatically between these 4 age groups. An increase in short chain acylcarnitines and a decrease in long chain acylcarnitine were observed during maturation. However, total carnitine content did not vary dramatically between the 4 age groups. The cellular concentrations of carnitine were calculated based on a cytosolic space of 2 ml·g dry wt^{-1} in heart muscle and assuming an equal distribution of carnitine between different subcellular compartments, as described by Idell-Wenger et al (1978). The myocardial total carnitine concentrations were: 1-day, 1.83 mM; 4-day, 1.36 mM; 10-day, 2.09 mM; 14-day, 2.03 mM. Free carnitine concentrations were: 1-day, 0.60 mM; 4-day, 0.55 mM; 10-day, 0.95 mM; 14-day, 0.95 mM.

3.3.2. CPT I Isoform Expression in the Newborn Heart

The expression of M-CPT I and L-CPT I in newborn rabbit hearts is shown in Figure 3.1. The expression of L-CPT I increased in the first 3 days after birth, but then returned to 1-day-old levels by day 10 (Figure 3.1A). In contrast M-CPT I expression was very low in 1-day, 3-day and 7-day old hearts, but increased in 10-day and 14-day old hearts (Figure 3.1B).

3.3.3. Kinetics of CPT I in Newborn Rabbit Heart Mitochondria

As shown in Figure 3.2, CPT I increased with increasing amount of protein in the assay medium. Shown in Figure 3.3 is the CPT I activity with increasing time of reaction. For kinetic analysis, CPT I activity was measured with the addition of 10 μg protein for 6 minutes incubation time.

The effects of various L-carnitine concentrations on CPT I activity in 1-day, 7-day, and 10-day-old rabbit hearts is shown in Figure 3.5. In heart mitochondria obtained from all 3 age groups, increasing L-carnitine concentration resulted in an increase in CPT I activity. In 1-day and 7-day old hearts, the Vmax for CPT I was similar despite a 10-fold increase in fatty acid oxidation during this period. However, in 10-day-old hearts, CPT I activity at higher L-carnitine concentrations was significantly greater then rates in 1-day and 7-day old hearts. This may reflect the increased expression of M-CPT I observed in these hearts compared to 1-day old hearts (Figure 3.2B).

Values for the Km for carnitine were obtained from Eadie Hofstee plots (right panels of Figure 3.4). Of interest was the observation that CPT I activity in mitochondria from 1-day and 10-day hearts appeared to express two different Km's for L-carnitine, whereas the best fit for 7-day old hearts was a single Km for L-carnitine.

3.3.4. Malonyl CoA Inhibition of CPT I in Rabbit Heart Mitochondria

We also determined whether changes in the sensitivity of CPT I to inhibition by malonyl CoA occur in the newborn heart. Malonyl CoA inhibition of CPT I activity was measured in mitochondria obtained from 1-day and 10-day old hearts (Figure 3.5). In these experiments, mitochondria were exposed to 200 µM L-carnitine. Addition of malonyl CoA resulted in a marked inhibition of CPT I activity (~95%). Despite this marked inhibition by malonyl CoA, there was no significant difference in the sensitivity of CPT I to malonyl CoA inhibition between 1-day and 10-day old hearts. The IC_{50} values for malonyl CoA were 4 nM and 5 nM in 1-day and 10-day-old rabbit hearts, respectively.

3.3.5. Malonyl CoA Degradation in the Newborn Heart

As shown in Figure 3.6, the activity of MCD increased in the heart in an age dependent manner. This increase in MCD activity was almost maximally increased in 7-day old hearts, a time period in which malonyl CoA levels dramatically decrease (Lopascuk et al 1994b), and fatty acid oxidation rates dramatically increase (Lopaschuk and Spafford 1990).

3.3.6. Fatty Acid Control of Glucose Oxidation in the Newborn Heart

While fatty acid oxidation increases dramatically following birth (probably due to a decrease in malonyl CoA levels), glucose oxidation (the other main source of acetyl CoA for the TCA cycle) does not increase during this period. Whether this is due to an increase in fatty acid inhibition of glucose oxidation, or due to a delayed maturation of the enzymes controlling glucose oxidation is not clear. We therefore determined what happens to the activity of myocardial pyruvate dehydrogenase (PDH), the rate-limiting enzyme for glucose oxidation, in the newborn period. We measured both the active phosphorylated form of PDH, as well as total PDH activity (Figure 3.7). A progressive age-dependent increase in active PDH and total PDH was observed between 1-day, 7-day and 10-day old hearts, suggesting that the capacity for glucose oxidation increases in the newborn period.

To determine if the increase in PDH was accompanied by an increase in glucose oxidation, isolated working hearts from 1-day and 7-day old hearts were used to measure glucose oxidation (Figure 3.8). Perfusion of hearts in the absence of fatty acids, a condition which measures the maximal capacity of the heart to oxidize glucose, showed that the capacity of the heart to oxidize glucose increases dramatically between 1-day and 7-days of age. This increase is consistent with the increase in PDH activity observed in Figure 3.7. However, if hearts were perfused with physiologically relevant levels of fatty acids, glucose oxidation rates were very low in both 1-day and 7-day old hearts (Figure 3.8). The dramatic increase in glucose oxidation observed in glucose-perfused hearts was completely prevented.

3.4. Discussion

The dramatic increase in fatty acid oxidation seen in the newborn heart has been attributed to an increase in L-carnitine levels and a switch from the L-CPT I isoform to the M-CPT I isoform (Brown et al 1995). However, since M-CPT I is more sensitive to inhibition by malonyl CoA this switch should actually lead to a decrease in fatty acid oxidation rates, not an increase as previously observed (Werner et al 1989, Lopaschuk et al 1990, Lopaschuk et al 1994b).

Results from this study demonstrated that tissue amounts of total carnitine did not change dramatically between age groups. Also, cellular concentrations of total carnitine were above the Km for either CPT I isoform expressed in the heart, making it unlikely that carnitine concentration was limiting for CPT I activity in the newborn period.

While the isoform expression pattern of CPT I did show changes in the developing rabbit heart, it is unlikely a switch in CPT I from the L-CPT I to M-CPT I isoform can explain the increase in fatty acid oxidation observed during this period. The maturation of fatty acid oxidation in the newborn heart occurs between 1-day and 7-days. This increase in fatty acid oxidation precedes the increase in M-CPT I expression we observed in these hearts.

It is possible that the increase in L-CPT I that occurred during the first few days following birth may have some role to play in the maturation of fatty acid oxidation in this period. However, this increase was transient, with L-CPT I mRNA expression appearing to decrease as M-CPT I expression increases. A recent paper by Cook et al suggests a differential regulation of the two CPT I genes in rat heart. These investigators showed a decline in the expression of the L-CPT I isoform during maturation and no change in the expression of M-CPT I (Cook et al 2001). These results differ somewhat from our results, although neither expression pattern could explain the observed increase in fatty acid oxidation seen following birth.

Of significance to this study is that even the highest Km values are below the concentrations of L-carnitine to which the newborn heart is exposed. Furthermore, there is no correlation between Km values for L-carnitine, and the ability of the newborn heart to oxidize fatty acids (Itoi and Lopaschuk 1996, Lopaschuk and Spafford 1990, Lopaschuk et al 1994b). As a result, increased myocardial concentrations of L-carnitine, changes in CPT I isoform expression and/or changes in the affinity of CPT I for L-carnitine are unlikely to be responsible for the dramatic increase in fatty acid oxidation seen in the newborn period.

It has been shown previously that electrical stimulation of isolated rat neonatal cardiac myocytes results in increased expression of M-CPT I and a greater sensitivity of CPT I to malonyl CoA inhibition (Xia et al 1996). We found no difference in CPT I activity or sensitivity to inhibition by malonyl CoA in 7-day old hearts compared to 1-day old hearts. This is not surprising since we did not observe increased M-CPT I expression until 10 days following birth. It is therefore unlikely that a switch in CPT I isoform is responsible for the elevated levels of fatty acid oxidation seen in 7-day-old hearts.

Although the sensitivity to malonyl CoA did not differ between these two age groups, previous studies from our laboratory have demonstrated that there is a dramatic reduction in malonyl CoA levels between 1-day old and 7-day old hearts (122.0 ± 8.3 nmol.g dry wt^{-1} in 1-day-old vs 1.4 ± 0.5 nmol.g dry wt^{-1} in 7-day-old rabbit hearts). This decrease in malonyl CoA is accompanied by a 10-fold increase in fatty acid oxidation (Lopaschuk et al 1994b). As a result, our data support the concept that it is a drop in malonyl CoA levels in the newborn heart that is probably the most important determinant of flux through CPT I and the increase in fatty acid oxidation in the newborn heart.

It has been demonstrated that acetyl CoA carboxylase is the key source of malonyl CoA synthesis in the heart (Saddik et al 1995), and that the decrease in malonyl CoA seen in the newborn heart is accompanied by a decrease in ACC activity (Lopaschuk et al 1994b). Since MCD is the key mechanism by which malonyl CoA is degraded in the heart (Dyck et al 1998, Dyck et al 2000, Hamilton and Saggerson 2000), we measured MCD activity changes in newborn rabbit hearts of different ages. As shown in Figure 3.6, the activity of MCD increased in the heart in an age dependent manner. This increase in MCD activity was almost maximally increased in 7-day old hearts, a time period in which malonyl CoA levels dramatically decrease (Lopaschuk et al 1994b), and fatty acid oxidation rates dramatically increase (Lopaschuk and Spafford 1990). This suggests that a drop in malonyl CoA and a decrease in malonyl CoA inhibition of CPT I may be one of the key mechanisms responsible for the maturation of fatty acid oxidation following birth.

The data from this study and our previous studies (Dyck et al 1998, Lopaschuk et al 1994b) suggest that an decrease in ACC activity and an increase in MCD activity are primarily responsible for the dramatic decrease in myocardial malonyl CoA levels in the newborn heart. The decrease in ACC activity in the newborn heart appears to be primarily due to an increased activity and expression of AMP-activated protein kinase (AMPK) in the newborn heart (Kantor et al 1999, Makinde et al 1997). AMPK activity/expression increases dramatically in the immediate newborn period, resulting in a phosphorylation and inactivation of ACC (Kantor et al 1999, Makinde et al 1997). The increase in MCD activity may be entirely due to increased MCD protein expression in the heart following birth (Dyck et al 1998). Alternatively, it is also possible that the increase in AMPK following birth may activate MCD. Saha et al (2000) have shown that AMPK can phosphorylate and activate skeletal muscle MCD in skeletal muscle. However, studies in adult heart (Dyck et al 1999) or in skeletal muscle (Habinowski et al 2001) did not show an activation of MCD by AMPK. Whether AMPK regulates MCD in the newborn heart remains to be determined.

Perfusion of hearts in the absence of fatty acids, a condition which measures the maximal capacity of the heart to oxidize glucose, showed that the capacity of the heart to oxidize glucose increases dramatically between 1-day and 7-days of age. This increase is consistent with the increase in PDH activity observed in Figure 3.7. However, if hearts were perfused with physiologically relevant levels of fatty acids, glucose oxidation rates were very low in both 1-day and 7-day old hearts (Figure 3.8). The dramatic increase in glucose oxidation observed in glucose-perfused hearts was completely prevented. This suggests that the newborn heart rapidly acquires the capability of oxidizing glucose, but that glucose oxidation is inhibited due to the increase in fatty acid oxidation observed during this period. Therefore, the decrease in malonyl CoA observed following birth not only accelerates fatty acid oxidation, but it also inhibits glucose oxidation during this period. This inhibition of glucose oxidation likely occurs as a result of an inhibition of pyruvate dehydrogenase that occurs as a result of the increase in fatty acid oxidation.

In summary, our results suggest that malonyl CoA has a key role in the maturation of fatty acid oxidation following birth. We demonstrate that a decrease in malonyl CoA, as opposed to changes in L-carnitine content, is responsible for the increase in fatty acid oxidation in the newborn heart. The decrease in malonyl CoA not only increases fatty acid oxidation, it also results in glucose oxidation rates remaining low in the newborn period. These results highlight the key role of malonyl CoA in the control of cardiac energy metabolism.

Table 3.1. Carnitine levels in newborn rabbit hearts

AGE (days)	**Free Carnitine** (nmol. g dry wt $^{-1}$)	**Short Chain Acyl Carnitine** (nmol. g dry wt $^{-1}$)	**Long Chain Acyl Carnitine** (nmol. g dry wt $^{-1}$)	**Total Carnitine** (nmol. g dry wt $^{-1}$)
1	1205 ± 148	690 ± 42	1771 ± 138	3666 ± 255 †
4	1149 ± 134	850 ± 14	728 ± 13 *	2727 ± 148
10	1894 ± 318	1955 ± 300 *†	336 ± 80 *	4186 ± 431 †
14	1853 ± 639	1967 ± 330 *†	250± 83 *	4070 ± 256 †

Carnitine concentrations were measured in newborn hearts as described in the "Experimental Procedures" section of this chapter.

Values are mean ± S.E. of 6 hearts in each group.

* significantly different from 1-day-old hearts

†significantly different from 4-day-old hearts

Figure 3.1. Expression of myocardial mRNA for the CPT I isoforms

mRNA expression was measured using RT-PCR ELISA as described in the "Experimental Procedures" section of this chapter.

A. L-CPT I

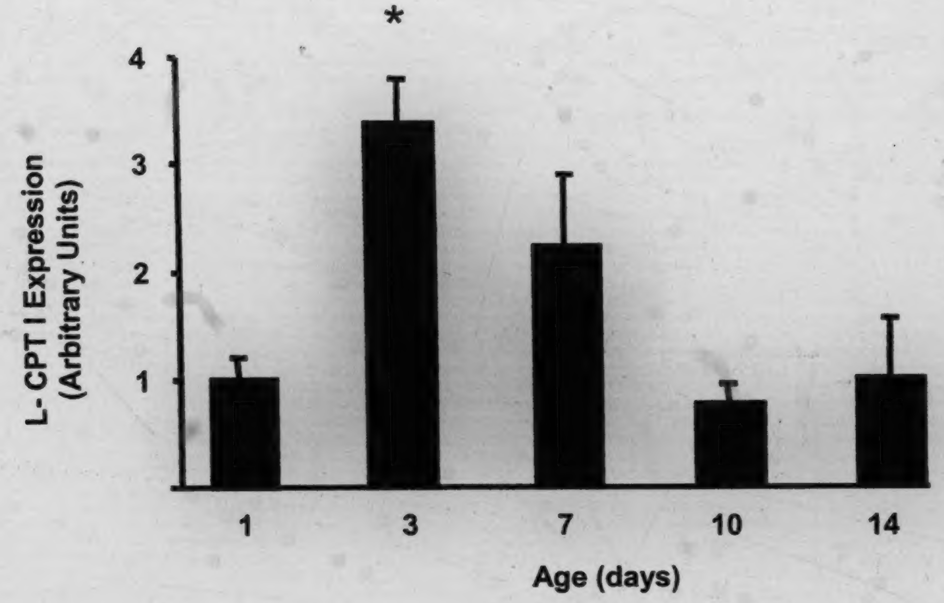
B. M-CPT I

Results are expressed relative to the level in 1-day-old hearts.

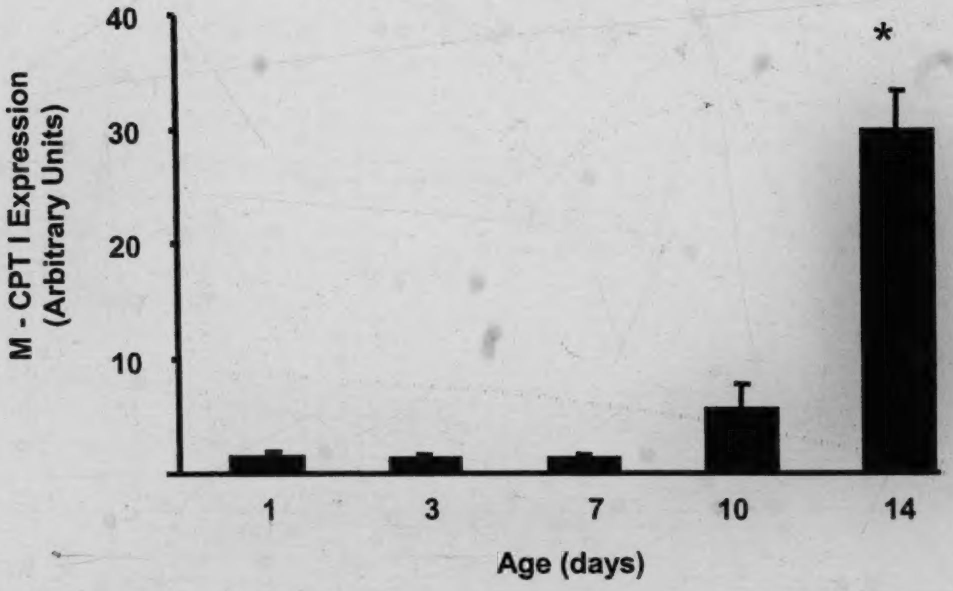
All experiments were carried out in duplicate, and results are mean determinations of 4 different rabbit hearts.

* significantly different from 1-day-old hearts.

A.


*
4
3
2
1
L- CPT I Expression (Arbitrary Units)
1
3
7
10
14
Age (days)

B.


*
40
30
20
10
M - CPT I Expression (Arbitrary Units)
1
3
7
10
14
Age (days)

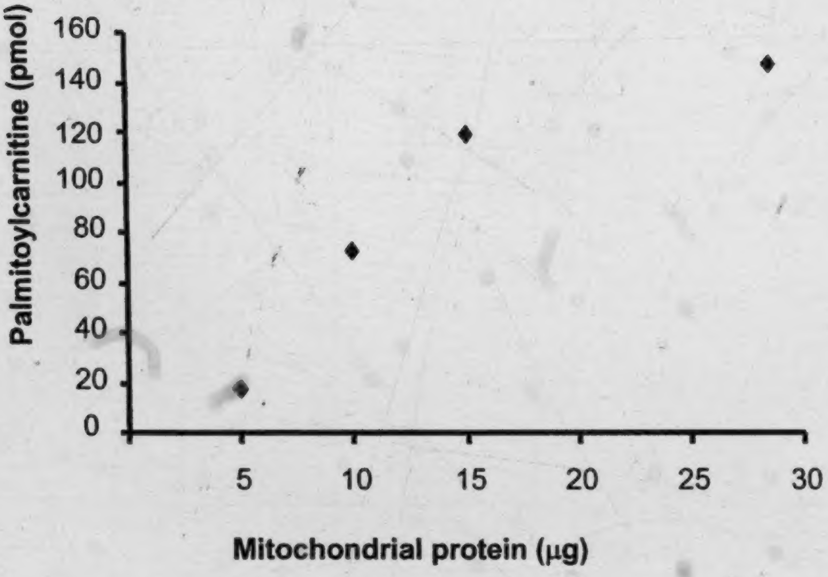


Figure 3.2. Effect of increasing the amount of mitochondrial protein on CPT I activity

CPT I activity was measured in the presence of 75 µM palmitoyl CoA, 200 µM carnitine, and increasing amounts of protein.
Values are mean of duplicates for each amount of protein.

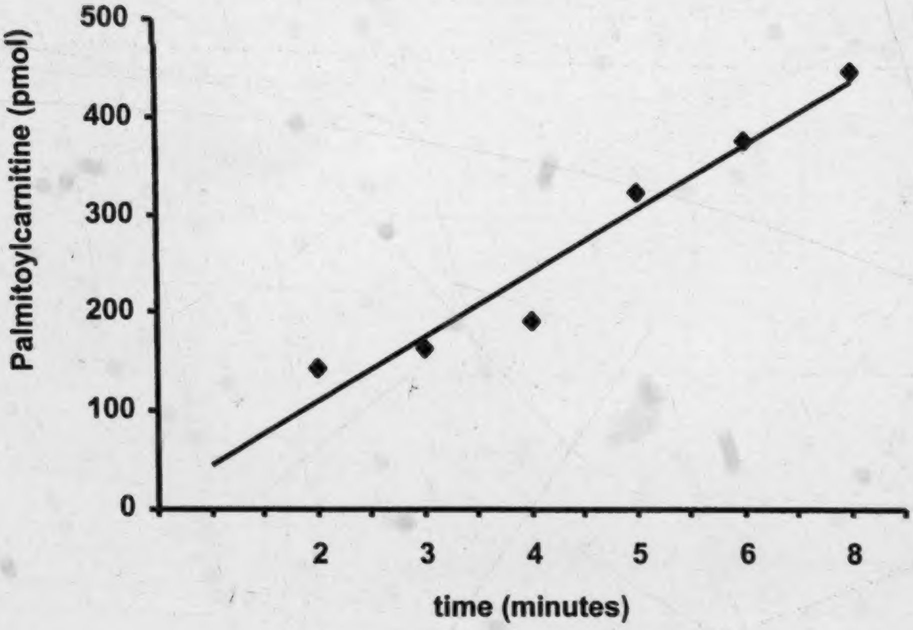


Figure 3.3. Time dependence of CPT I Activity

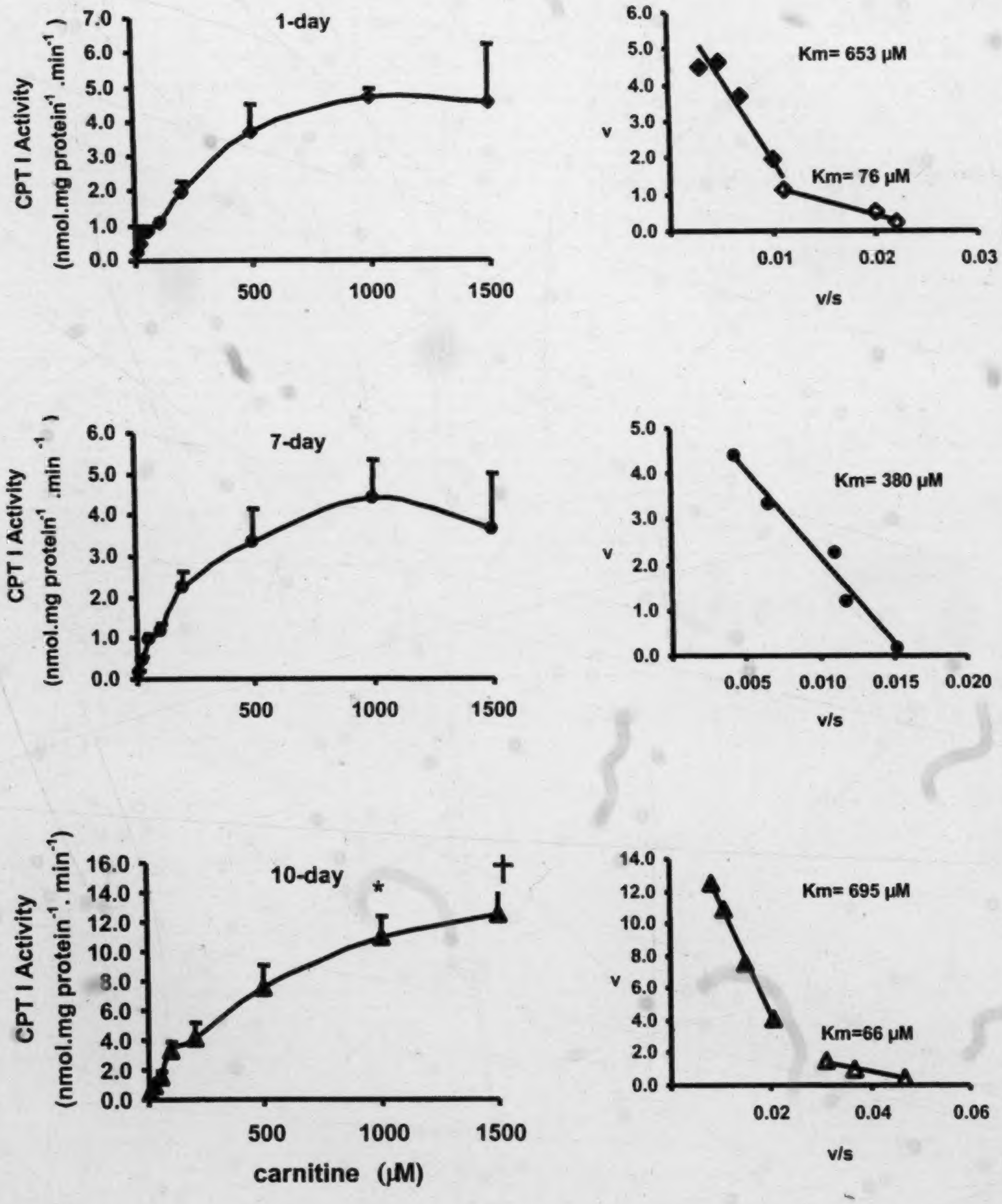
CPT I activity was measured using 10 μg mitochondrial protein in the presence of 75 μM palmitoyl CoA and 200 μM carnitine.

Values are mean of duplicates for each time point.

Figure 3.4. CPT I Activity and corresponding Eadie Hofstee Plots at different ages

CPT I activity was measured in mitochondria prepared from rabbit hearts as described in the "Experimental Procedures" section. Values are mean ± S.E. of 5 experiments in 1-day-old rabbits (two hearts were pooled to increase the mitochondrial yield), 5 hearts for 7-day, and 8 for 10-day-old hearts.
Eadie Hofstee Plots were used to determine the Km for carnitine. Plots are given besides each curve for each age group.

* significantly different from 1-day-old hearts at same carnitine concentration.
† significantly different from 7-day-old hearts at same carnitine concentration.


1-day
CPT I Activity
(nmol.mg protein^{-1} .min^{-1})
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
500
1000
1500
6.0
5.0
4.0
3.0
2.0
1.0
0.0
v
Km= 653 µM
Km= 76 µM
0.01
0.02
0.03
v/s
7-day
CPT I Activity
(nmol.mg protein^{-1} .min $^{-1}$)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
500
1000
1500
5.0
4.0
3.0
2.0
1.0
0.0
v
Km= 380 µM
0.005
0.010
0.015
0.020
v/s
10-day
*
†
CPT I Activity
(nmol.mg protein^{-1}. min^{-1})
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
500
1000
1500
carnitine (µM)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
v
Km= 695 µM
Km=66 µM
0.02
0.04
0.06
v/s

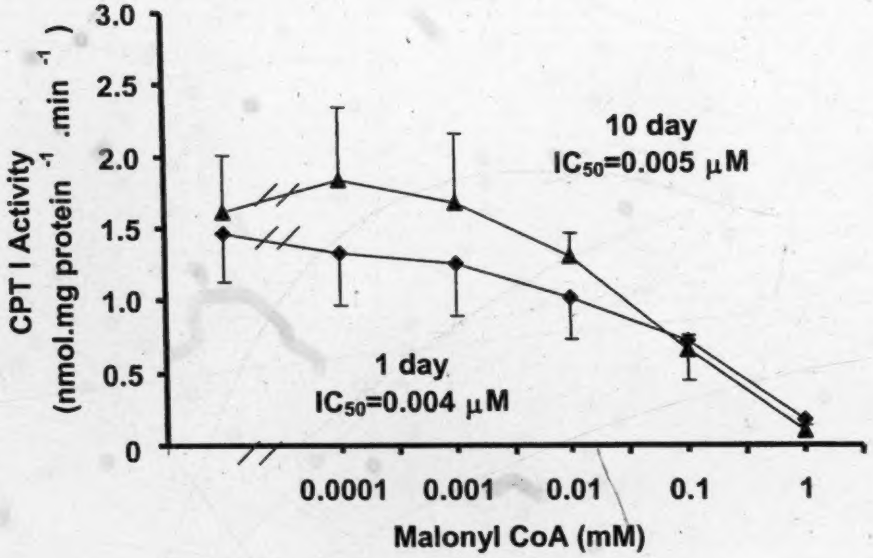


Figure 3.5. Malonyl CoA inhibition of CPT I activity

CPT I activity was measured in mitochondria of newborn rabbit heart as described in Chapter 2.

Values are mean ± S.E. of 8 experiments in 1-day-old, and 8 hearts in 10-day-old hearts.

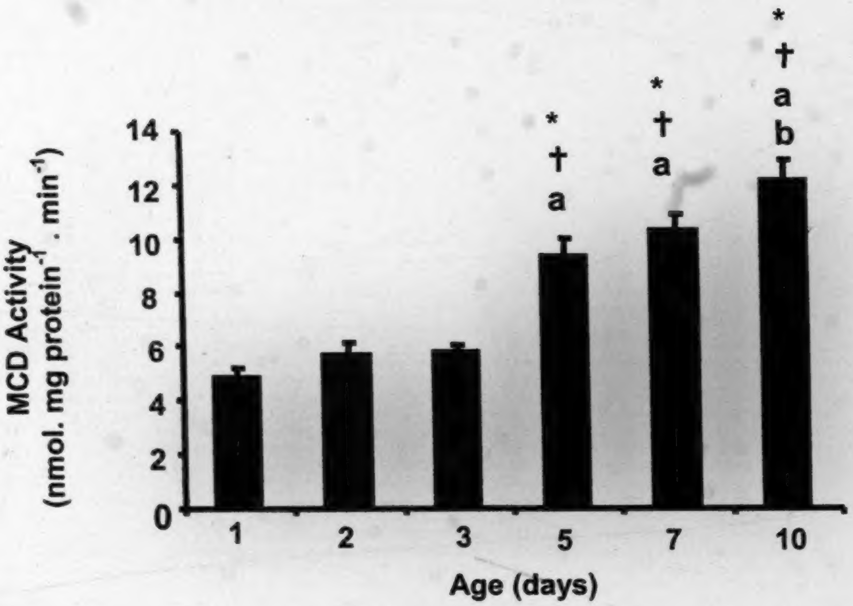


Figure 3.6. Malonyl CoA decarboxylase (MCD) activity in newborn rabbit hearts

MCD activity was measured in newborn rabbit hearts as described in Chapter 2. Values are mean ± S.E. of 5 hearts in each group.

* significantly different from 1-day-old hearts

† significantly different from 2-day-old hearts

a significantly different from 3-day-old hearts

b significantly different from 5-day-old hearts

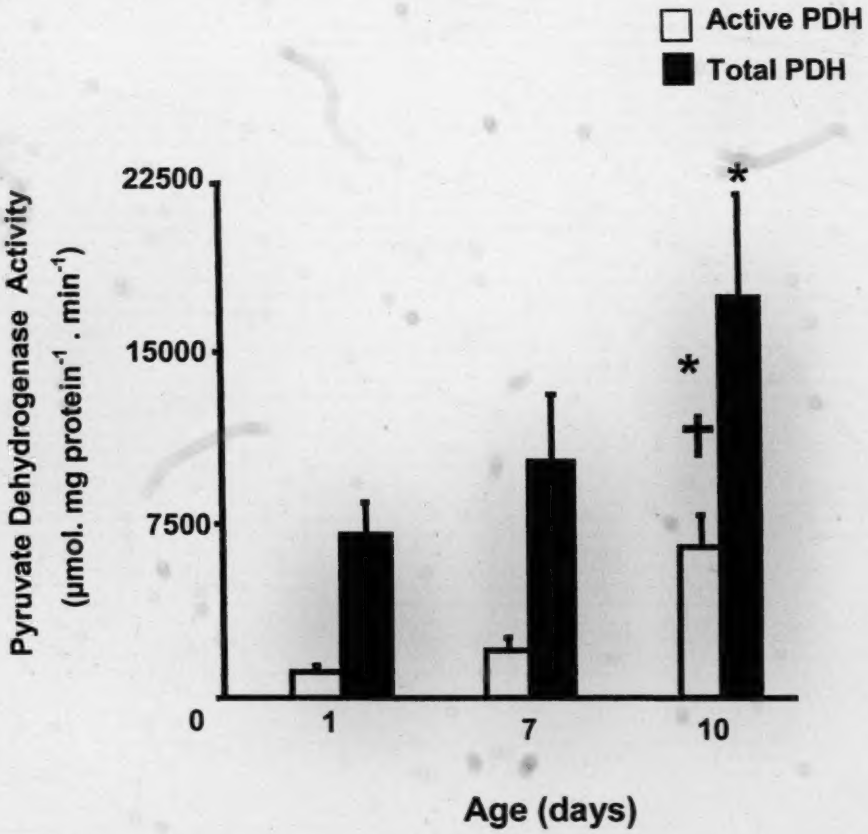


Figure 3.7. Pyruvate dehydrogenase activity in newborn rabbit hearts

PDH activity was measured in measured rabbit hearts as described in Chapter 2. Values are mean ± S.E. of 4 hearts in each group.

* significantly different from 1-day-old hearts

† significantly different from 7-day-old hearts

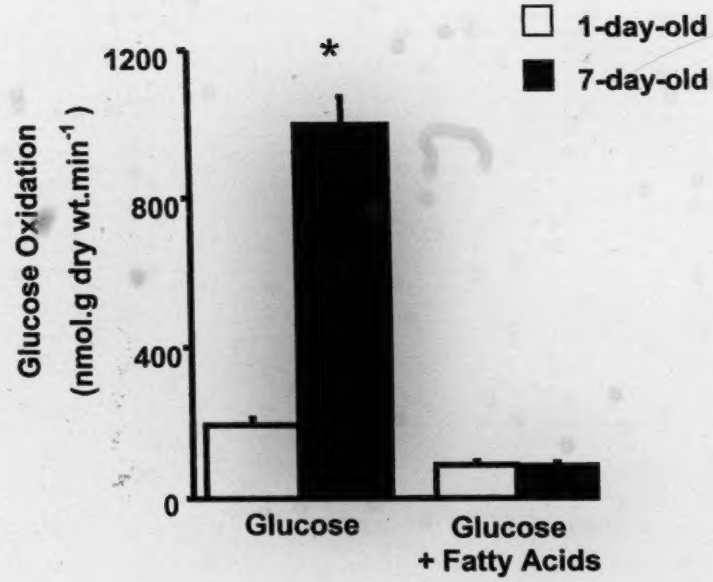


Figure 3.8. Glucose oxidation rates measured in 1-day and 7-day-old newborn rabbit hearts perfused in the presence and absence of 0.4 mM palmitate

Glucose oxidation was measured in newborn rabbit hearts as described in Chapter 2.
Values are mean ± S.E. of 6 hearts in each group.

* significantly different from 1-day-old hearts.

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Chapter 4

Malonyl CoA Control of Fatty Acid Oxidation in the Newborn Heart in Response to Increased Fatty Acid Supply

Manuscript in preparation to be submitted to Journal of Molecular and Cellular Cardiology:

Besikci, A.O., Sambandam, N., Lopaschuk, G.D. *Malonyl CoA Control of Fatty Acid Oxidation in the Newborn Heart in Response to Increased Fatty Acid Supply*

4.1. Introduction

The heart has very high demand for energy in the entire organism. Energy in the form of ATP is primarily supplied from the oxidation of fatty acids and glucose in the adult heart. In the fetal heart on the other hand, lactate oxidation and glycolysis are preferred sources for ATP production (Lopaschuk and Spafford 1990, Lopaschuk et al 1991, Werner and Sicard 1987). As the newborn heart matures, fatty acid oxidation increases and becomes the dominant oxidative substrate for the heart in most mammalian species. The mechanism for this switch in energy substrate preference is a combination of changes of postnatal nutrition and environment, as well as direct subcellular changes within the myocardium (reviewed in Girard et al 1992).

The concentration of fatty acids in the blood or perfusate is a major determinant of the extent of myocardial fatty acid oxidation. For example, increasing fatty acid supply to the adult rat heart has been shown to increase myocardial fatty acid oxidation (Longnus et al 2001). High levels of fatty acids have been reported post-surgery in the plasma of infants undergoing cardiac bypass operation to correct congenital heart defects (Lopaschuk et al 1994a). How the newborn heart responds to increased fatty acid supply remains to be determined.

A key site of control of fatty acid oxidation is at the level of transport of the fatty acids into the mitochondria. CPT I catalyzes the formation of long chain fatty acylcarnitines from fatty acids and free carnitine. Malonyl CoA, the substrate for CPT I in hepatic lipogenesis, is also a potent physiological inhibitor of the enzyme (reviewed in McGarry and Foster 1980). In many physiological (increase in fatty acid oxidation in the postnatal period) and pathological situations (diabetes, hypertrophy, ischemic heart disease) as well as experimental models, alterations in tissue levels of malonyl CoA have been shown to regulate the fatty acid oxidation (reviewed in Dyck and Lopaschuk 2002). The regulation of malonyl CoA levels by AMPK and ACC has been discussed in detail in Section 1.4.

The supply of acetyl CoA to ACC is another important mechanism by which malonyl CoA synthesis can be increased or decreased to address the metabolic demands of the heart (Saddik et al 1993). Moreover, ACC280 is primarily regulated by acetyl CoA supply to the enzyme, as opposed to regulation by phosphorylation or by the allosteric activator citrate. Increases in the intramitochondrial acetyl CoA/CoA ratio caused either by decreases in metabolic demand or increases in acetyl CoA will increase the transfer of acetyl groups from the mitochondria into the cytosol via carnitine acetyltransferase-carnitine acetyltranslocase system that leads to the formation of acetylcarnitine and acetyl CoA in the cytosol (Lysiak et al 1986, Lysiak et al 1988). This increase in cytosolic acetyl CoA stimulates ACC-mediated synthesis of malonyl CoA, which results in decreased fatty acid oxidation (Saddik et al 1993).

In this study, we examined if the tissue levels of malonyl CoA will further decrease to relieve the inhibition on CPT I when myocardium is exposed to high concentrations of long-chain fatty acids in newborn rabbit heart. We then tested the contribution of the enzymes that regulate tissue levels of malonyl CoA and acetyl CoA.

4.2. Experimental Procedures

Isolated Working Heart Preparation and Perfusion

Seven-day-old rabbits were injected with 60 mg/kg i.p. sodium pentobarbital. Hearts were removed and placed in ice-cold Krebs-Henseleit solution. The aortae were quickly cannulated, and the hearts were perfused via the aorta with oxygenated Krebs-Henseleit solution. During this initial aortic perfusion, hearts were trimmed of excess tissue and the left atria were cannulated.

Hearts were then switched to the working mode and perfused with oxygenated KH solution containing 11 mM [U-^{14}C]glucose and 0.4 mM (low-fat) or 1.2 mM (high-fat) [9,10-^{3}H]palmitate bound to 3% bovine serum albumin, 2.5 mM calcium, and 100 μU/ml insulin. The hearts were perfused aerobically for 40 min as described in Section 2.2.2. The samples for the measurement of palmitate oxidation and glucose oxidation were collected at 10-, 20-, 30-, and 40 min time points as described in Section 2.3.1 and Section 2.3.2. Metabolic values were normalized for heart mass (dry wt).

At the end of the perfusions, hearts were quickly clamped tongs that had been cooled to the temperature of liquid nitrogen. Frozen ventricular tissue was weighed, powdered (using a mortar and pestle that had been cooled to the temperature of liquid nitrogen), and stored in cryovials at -80°C until use.

Determination of CoA Esters

CoA esters were extracted from frozen ventricular tissue using 6% perchloric acid, and were then quantified using high performance liquid chromatography (HPLC) as described in Section 2.7.

Western Blot Analysis of Ser79-phosphorylated ACC

Heart homogenates (30 μg) were subjected to SDS-polyacrylamide gel electrophoresis using standard procedures as described in Section 2.6. Membranes were probed with a rabbit antibody against Ser79-phosphorylated ACC (Upstate Biotechnology, 1: 1000 in 1% milk in TBS with 0.05% Tween 20). Secondary peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz, 1:1000 in 1% milk in TBS with 0.05% Tween 20) followed by ECL Western Blotting kit was used to visualize ACC.

Enzyme Activity Assays

AMPK, ACC, and MCD activities were determined in perfused hearts that have been frozen at the and of the 40 min protocol as described in Section 2.4.

4.3. Results

4.3.1. Mechanical Function of the Hearts

Mechanical function of the low-fat and high-fat-perfused hearts is summarized in Table 4.1. Cardiac function was stable over the duration of the perfusion (data not shown). Interestingly, heart rate of the high-fat group was lower, and peak systolic pressure was higher compared to low-fat group. However, these changes with high-fat exposure did not affect cardiac work (cardiac output x peak systolic pressure).

4.3.2. The Rates of Fatty Acid Oxidation and Glucose Oxidation

Rates of fatty acid oxidation are shown in Figure 4.1. Hearts perfused with 1.2 mM palmitate (high-fat) exhibited significantly higher rates of fatty acid oxidation than those perfused with 0.4 mM palmitate concentration (low-fat).

Figure 4.2 shows rates of glucose oxidation in low-fat and high-fat-perfused hearts. Glucose oxidation rates were significantly lower in high-fat-perfused hearts compared to low-fat-perfused hearts.

Using these glucose and fatty acid oxidation rates, we calculated the contribution of the two pathways to acetyl CoA production in low-fat and high-fat-perfused hearts (Figure 4.3). Acetyl CoA production was calculated using a value of 2 moles of acetyl CoA produced per mole glucose oxidized, and 8 moles of acetyl CoA produced per mole palmitate oxidized. In low-fat-perfused hearts, fatty acid oxidation was the major source of acetyl CoA production (i.e., fatty acid oxidation provided 86% of the overall acetyl CoA production) with glucose oxidation providing 14%. In high-fat-perfused hearts, a further increase was observed in the contribution of fatty acid to acetyl CoA production; 98.5% of overall acetyl CoA was supplied by fatty acid oxidation.

4.3.3. Tissue Levels of Malonyl CoA and Acetyl CoA

Figure 4.4 shows levels of malonyl CoA and acetyl CoA measured in hearts frozen at the end of each perfusion. Malonyl CoA levels were significantly lower (Figure 4.4A) and acetyl CoA levels were higher (Figure 4.4B) in high-fat perfused hearts compared to low-fat-perfused hearts.

4.3.4. Activities of AMPK, ACC, and MCD

The activities of the enzymes that regulate the production and degradation of malonyl CoA were examined next. The time course and protein dependence were determined for all three enzymes. Activities of the enzymes increased with increasing amount of protein in the assay medium, and increasing time of the reactions (data not shown). AMPK activity was unaltered by increasing palmitate concentration from 0.4 to 1.2 mM (Table 4.2). Since AMPK phosphorylates and inactivates ACC, the phosphorylation status of ACC at Ser79 was examined. The amount of Ser79-phosphorylated ACC protein was not different between the low-fat or high-fat-perfused hearts. A representative western blot is shown in Figure 4.5A. The densitometric analysis of the western blot is shown in Figure 4.5B. Similarly, no significant differences were observed between the two groups in the activities of ACC and MCD (Table 4.2).

4.4. Discussion

Several studies have shown that increasing fatty acid supply to the heart increases myocardial fatty acid oxidation (Neely and Morgan 1974, Saddik and Lopaschuk 1991, Longnus et al 2001). The increase in fatty acid oxidation is likely the result of an increased uptake of fatty acids into the myocardium. In order to enter the mitochondria for further metabolism, however, CoA esters of the long-chain fatty acids must overcome the inhibition of the gate-keeper enzyme on the mitochondrial membrane, CPT I.

In a recent study, isolated adult rat hearts were exposed to different concentrations of long-chain (palmitate) and medium-chain (octanoate) fatty acid (Longnus et al 2001). Hearts perfused at a high fatty acid concentration (both long- and medium-chain fatty acids) exhibited higher fatty acid oxidation than those perfused with low levels of fatty acids. However, the increase in high-palmitate group was not accompanied by a decrease in malonyl CoA. The authors concluded that the supply of acetyl CoA adequately met the demand of the Krebs' Cycle for acetyl CoA. Although glucose oxidation was not measured in that study, glycolysis was shown to decrease ~30% which may indicate the remaining contribution of PDH-mediated acetyl CoA production in the high-palmitate-perfused group.

In this study, the increase in fatty acid oxidation in high-fat-perfused hearts was accompanied by a decrease in malonyl CoA levels. Moreover, the levels of acetyl CoA were higher in high-fat group compared to the low-fat group. Malonyl CoA is synthesized by acetyl CoA carboxylase (ACC) from acetyl CoA. Two major isoforms of ACC have been identified, a 265-kDa (ACC265, ACC1 or ACCα) and a 280-kDa (ACC280, ACC2 or ACCβ) isoform. Both isoforms are expressed in the heart (Abu-Elheiga et al 1997, Lopaschuk et al 1994b). ACC appears to be a "substrate-driven" enzyme due to low tissue levels of its substrate (acetyl CoA) compared with the Km of the enzyme for this substrate (Poirier et al 2002). This concept was supported in other studies where increases in the amount of acetyl CoA available to ACC lead to the increased synthesis of malonyl CoA (Saddik et al 1993, Longnus et al 2001). In our study, the levels of malonyl CoA were lower despite higher levels of acetyl CoA in high-fat-perfused hearts compared to low-fat-perfused hearts. However, the increase in acetyl CoA is probably related to the increase in the mitochondrial portion of the acetyl CoA as a result of the increase in palmitate oxidation (2 molecules of acetyl CoA are produced by the oxidation of one molecule of glucose, whereas 8 molecules of acetyl CoA is produced by the oxidation of one molecule of palmitate). Why is the excess acetyl CoA not transferred out of the mitochondria via the carnitine actyltransferase/carnitine acetyltranslocase system? Saddik et al (1993) showed that the stimulation of PDC by dichloroacetate (DCA) increased the levels of both acetyl CoA and malonyl CoA in rat hearts. The authors suggested that an increase in acetyl CoA supply from PDC stimulates ACC activity. Furthermore, Lysiak et al (1986) showed that most of acetyl CoA generated from pyruvate by PDC is readily accessible to carnitine acetyltransferase while that generated from β-oxidation is more available to the Krebs' Cycle. Unfortunately, technical limitations do not allow for an accurate measurement of cytosolic versus intramitochondrial acetyl CoA levels in our perfused hearts nor the source of acetyl CoA. Although highly speculative, we suggest that the increase in acetyl CoA reflects an increase in the mitochondria that originates from increased β-oxidation in high-fat-perfused hearts.

The levels of malonyl CoA are regulated at the level of both production and degradation. Previous studies suggested that an increase in AMPK activity and resulting decrease in ACC activity was involved in situations in which cardiac fatty acid oxidation increases, such as the fetal to newborn transition (Makinde et al 1997, Lopaschuk et al 1994b), and reperfusion of the ischemic heart (Kudo et al 1995). The decrease in ACC activity resulted in a decrease in malonyl CoA levels in these studies. Studies from our laboratory suggested that MCD is involved in the decrease in malonyl CoA levels and in the increase in fatty acid oxidation. For instance, MCD activity increases in the fetal to newborn transition (Dyck et al 1998, Onay-Besikci et al 2002), and in diabetes (Sakamoto et al 2000). In this study, we did not observe a change in the activities of the known regulatory enzymes of tissue malonyl CoA levels. However, we measured the activities of these enzymes (AMPK, ACC, and MCD) in whole tissue homogenates, and not in subcellular fractions such as cytosol or mitochondria. This might have limited the possibility to detect alterations in the activities of these enzymes. Indeed, we did see a slight but insignificant increase in MCD activity in the high-fat-perfused hearts.

Another possibility is that the affinity of malonyl CoA increases for MCD in high-fat-perfused hearts. To our knowledge, extensive kinetic studies have not been performed in MCD for malonyl CoA in the heart. If in fact malonyl CoA has a higher affinity for MCD in high-fat-perfused hearts, the levels of malonyl CoA would be expected to decrease without an increase in the maximal enzyme rate.

In summary, we report that increasing fatty acid supply to newborn hearts results in an increase in fatty acid oxidation rates. This increase is accompanied by a decrease in tissue levels of malonyl CoA. Detailed kinetic analysis of MCD and subcellular localization studies are needed to determine the exact mechanism of the in malonyl CoA levels.

Table 4.1. Mechanical function of isolated 7-day-old working hearts perfused with low or high fatty acid concentrations

	Low-fat (0.4 mM)	High-fat (1.2 mM)
Heart rate (beats/ min)	213 ± 3	204 ± 3
Peak pressure (mm Hg)	57 ± 1	59 ± 1
Heart rate x peak pressure (beats/min x mmHg x 10^{-3})	20 ± 2	21 ± 1

Isolated working hearts were perfused with 0.4 mM (low-fat) or 1.2 mM (high-fat) palmitate bound to 3% bovine serum albumin at an 7.5 mm Hg left atrial preload and an 35 mm Hg aortic afterload.

Values are means ± SE of 6 hearts in low-fat, and 7 hearts in high-fat group.

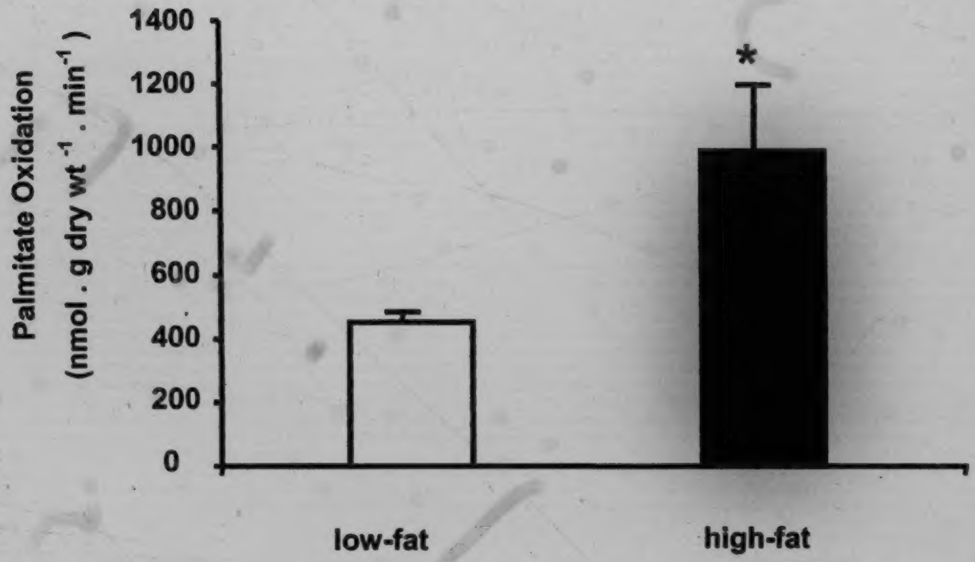


Figure 4.1. Fatty acid oxidation rates in 7-day old rabbit hearts

Isolated working hearts were perfused with 0.4 mM (low-fat) and 1.2 mM (high-fat) palmitate.

Values are means ± SE of 6 hearts in low-fat and 7 hearts in high-fat group.

* significantly different from low-fat group.

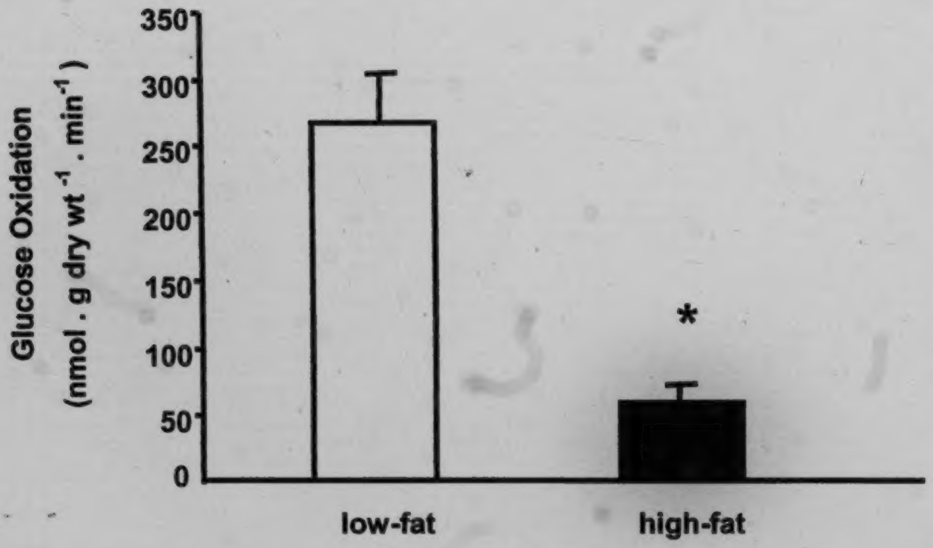


Figure 4.2. Glucose oxidation rates in 7-day-old rabbits

Isolated working hearts were perfused with 0.4 mM (low-fat) and 1.2 mM (high-fat) palmitate.

Values are means ± SE of 6 hearts in low-fat and 7 hearts in high-fat group.

* significantly different from low-fat group.

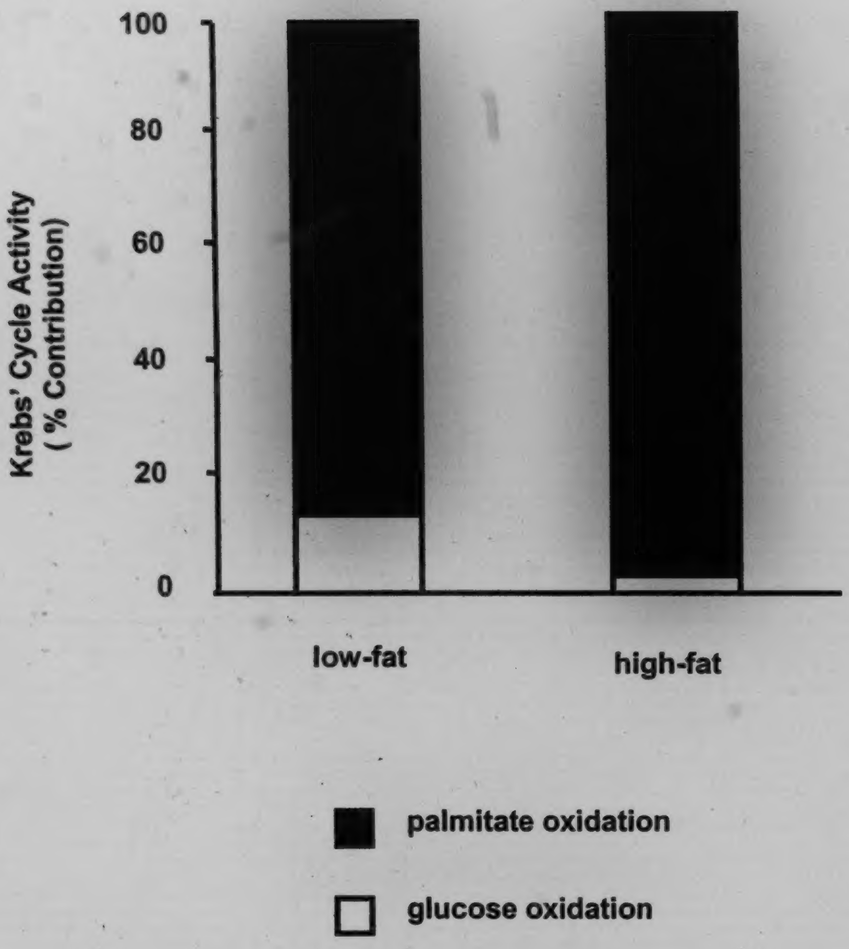


Figure 4.3. The percentage of contribution of fatty acid and glucose oxidation to overall acetyl CoA production in 7-day-old rabbit hearts

The hearts were perfused with 0.4mM (low-fat) and 1.2mM (high-fat) palmitate. Acetyl CoA production by each pathway was calculated from fatty acid and glucose oxidation rates (Figure 4.1, and Figure 4.2, respectively), using a value of 2 moles of acetyl CoA produced per mole of glucose oxidized (white bars), and 8 moles of acetyl CoA per mole of palmitate oxidized (black bars).

Figure 4.4. Tissue contents of CoA esters in hearts perfused with 0.4 mM (low-fat) and 1.2 mM (high-fat) palmitate

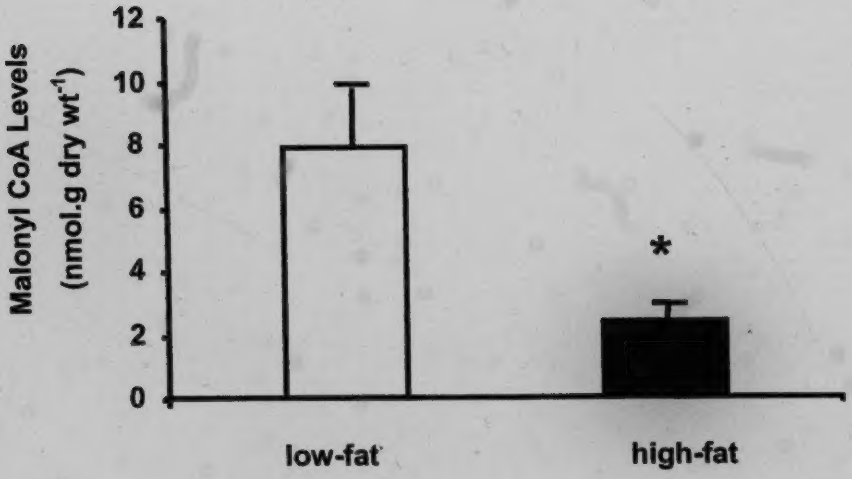
A. Levels of malonyl CoA

B. Levels of acetyl CoA

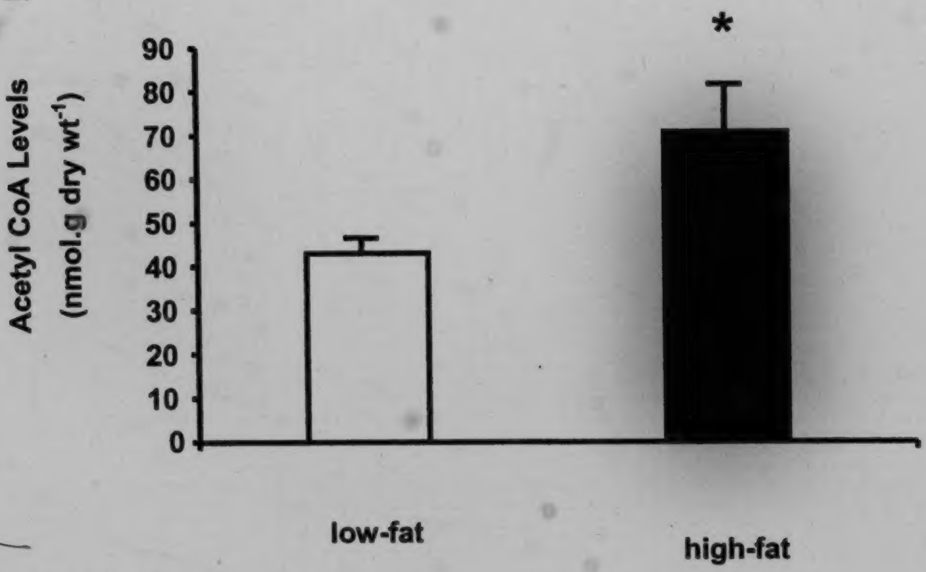
Values are means ± SE of 6 hearts in low-fat and 8 hearts in high-fat group.

* significantly different from low-fat group.

A.


Malonyl CoA Levels
(nmol.g dry wt^{-1})
12
10
8
6
4
2
0
*
low-fat
high-fat

B.


Acetyl CoA Levels
(nmol.g dry wt^{-1})
90
80
70
60
50
40
30
20
10
0
*
low-fat
high-fat

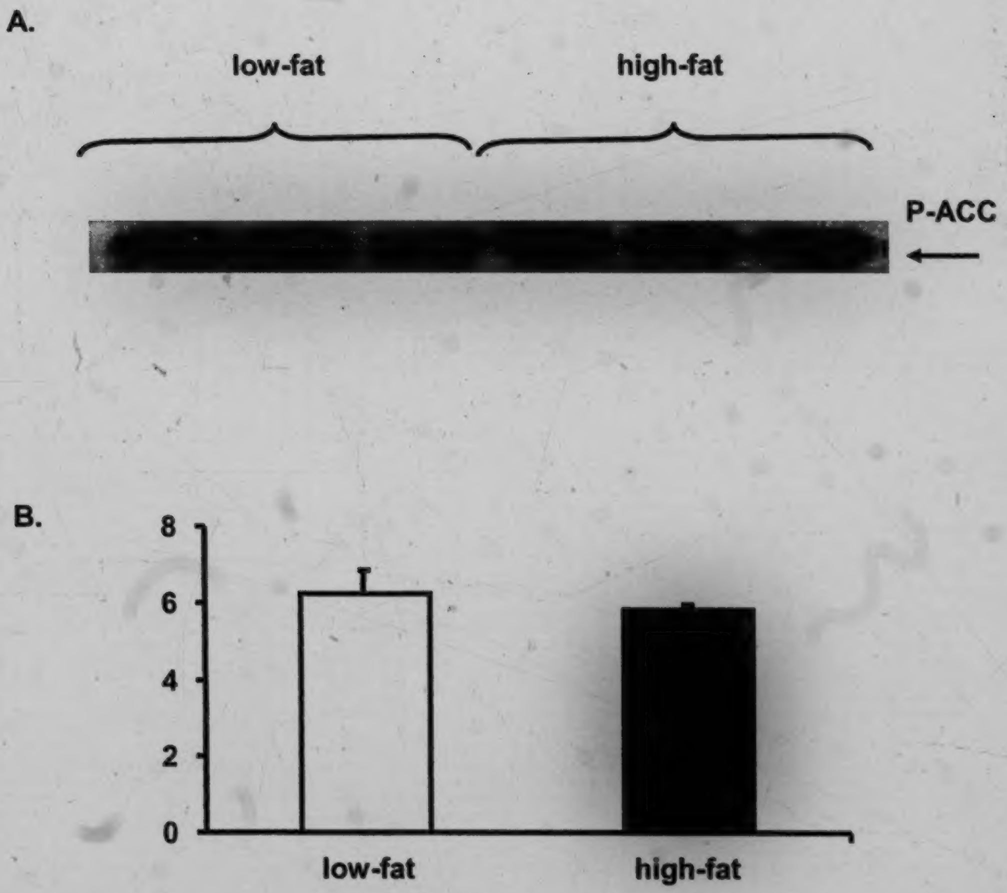


Figure 4.5. The expression of Ser79-phosphorylated ACC and ACC activity in 7-day-old rabbit hearts

A. Immunoblot analysis of Ser79-phosphorylated ACC in 7-day-old hearts perfused with 0.4 mM (low-fat) or 1.2 mM (high-fat) palmitate.

Each lane represents one heart.

B. Densitometric analysis of the immunoblot shown in Figure 4.5A.

Values in the bar graph are mean ± S.E.

Table 4.2. ACC, MCD, and AMPK activities in hearts perfused with low or high fatty acid concentrations

	Low-fat (0.4 mM)	High-fat (1.2 mM)
ACC (nmol . mg protein^{-1} min^{-1})	0.36 ± 0.06	0.47 ± 0.11
MCD (nmol . mg protein^{-1}. min^{-1})	8.25 ± 0.57	10.07 ± 0.91
AMPK (nmol . mg protein^{-1} . min^{-1})	5.17 ± 0.12	4.82 ± 0.18

Values are means ± SE of 6 or 8 hearts in each group.

ACC, acetyl CoA carboxylase

MCD, malonyl CoA decarboxylase

AMPK, AMP-activated protein kinase

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Chapter 5

gAd-Globular Head Domain of Adiponectin Increases Fatty Acid Oxidation in Newborn Rabbit Hearts

A paper is presently being prepared for submission to "Journal of Biological Chemistry":

Besikci, A.O., and Lopaschuk, G.D. *gAd-Globular Head Domain of Adiponectin Increases Fatty Acid Oxidation in Newborn Rabbit Hearts*

5.1. Introduction

Adiponectin is an adipocyte-derived polypeptide hormone of ~30-kDa molecular weight (Scherer et al 1995). It has a signal sequence at the amino terminus, a small nonhelical region, a stretch of repeated collagens and a C-terminal globular head domain (gAd) that makes up for the majority of the protein (Nakano et al 1999). Recently, a proteolytic cleavage product of adiponectin has been shown to increase fatty acid oxidation in muscle and cause weight loss in mice (Fruebis et al 2001). Yamauchi et al (2002) demonstrated that gAd and full-length adiponectin stimulated 5'-AMP-activated protein kinase (AMPK) in skeletal muscle. The authors showed that this activation increased the phosphorylation of acetyl CoA carboxylase (ACC), fatty acid oxidation, glucose uptake and lactate production in C2C12 myocytes. When administrated *in vivo*, both full-length adiponectin and gAd stimulated AMPK and increased the phosphorylation of ACC in mouse soleus muscle (Yamauchi et al 2002). Moreover, stimulation of AMPK with full-length adiponectin reduced gluconeogenesis in the liver and plasma glucose in the same study (Yamauchi et al 2002). In agreement with these findings, Tomas et al (2002) reported that gAd stimulates fatty acid oxidation in skeletal muscle by activating AMPK and inactivating ACC.

Cardiac energy in the form of ATP is primarily supplied from the oxidation of fatty acids and glucose in the adult heart. In contrast, in the fetal heart, lactate oxidation and glycolysis are preferred sources for ATP production (reviewed in Girard et al 1992, Lopaschuk et al 1992, Makinde et al 1998). As the newborn heart matures, fatty acid oxidation increases and becomes the dominant oxidative substrate for the heart. The mechanism for this switch in energy substrate preference is due to a combination of changes of nutrition and environment, as well as direct subcellular changes within the myocardium.

Plasma insulin decreases and glucagon increases in the immediate postnatal period in most species including humans, rats, rabbits, sheep, and pigs (Bassett and Alexander 1971, Blazquez et al 1974). The perinatal decrease in insulin and increase glucagon have been suggested to be the result of the stress of birth and through the activation of the sympathetic nervous system (Girard et al 1973, Padbury et al 1981). This hormonal environment is persistent throughout the suckling period, and is thought to be related to the high fat and low carbohydrate content of the diet that is consumed by the newborn. What happens to adiponectin following birth is not known.

Malonyl CoA is a potent endogenous inhibitor of mitochondrial fatty acid oxidation. Malonyl CoA is synthesized in the heart by ACC, the activity of which decreases after birth (Lopaschuk et al 1994). ACC is phosphorylated and inactivated by AMPK (reviewed in Hardie and Carling 1997), the expression and activity of which also increases in the heart after birth (Makinde et al 1997). Malonyl CoA is degraded in the heart by decarboxylation to acetyl CoA by malonyl CoA decarboxylase (MCD) (Dyck et al 1998), the activity of which increases after birth (Dyck et al 1998, Onay-Besikci et al 2003). We have suggested that the simultaneous increase in MCD, and decrease in ACC and malonyl CoA contribute to the increase in fatty acid oxidation in the newborn period (Makinde et al 1997). Whether changes in gAd or gAd control of AMPK contributes to the increase in fatty acid oxidation has not been determined.

The objective of this study was to examine the role of adiponectin and gAd in the increase in fatty acid oxidation in the newborn heart. We also examined the interaction between insulin and gAd in the regulation of fatty acid oxidation in the newborn heart.

5.2. Experimental Procedures

Experimental Animals

Hearts from anesthetized 1-day-old New Zealand White rabbits were removed and placed in ice-cold Krebs-Henseleit solution as described in Chapter 2. Blood was collected from 1-day, 7-day, and 6-week-old rabbits and plasma was separated by centrifugation (10,000 x g for 10-min).

Western Blot Analysis of Adiponectin in Plasma

Plasma samples of 1-day, 7-day, and 6-week-old rabbits were subjected to SDS-polyacrylamide gel electrophoresis as described in Chapter 2. Following transfer onto nitrocellulose, bands were probed with a polyclonal antibody directed against adiponectin (Chemicon International, 1:2000 dilution in Tris-buffered saline with 0.05% Tween 20 in 1% non-fat milk). After reaction with a secondary antibody (anti-mouse IgG-HRP), the blots were visualized with chemiluminescent detection using an ECL Western blotting detection kit. Adiponectin concentrations in samples were then calculated from a linear standard curve generated by increasing amounts of mouse recombinant adiponectin on the same gel.

Recombinant Protein Production and Protein Characterization

Recombinant adiponectin was produced by following the protocol described in Fruebis et al (2001) except that cDNA was cloned in pET-30a (Novagen), and Chelating Sepharose Fast Flow column was used to isolate the N-terminal His_6-tagged fusion protein from the lysed bacterial pellet. To detect the full-length adiponectin, the end product was separated by SDS-PAGE, and transferred onto nitrocellulose paper by standard procedures. HRP-conjugated anti-His-tag antibody (Santa Cruz) and mouse anti-adiponectin antibody raised against the globular head domain (Chemicon) followed by HRP-conjugated anti-mouse IgG (Santa Cruz) was used to visualize the proteins.

Heart Perfusions

Isolated hearts obtained from 1-day-old hearts were perfused in Langendorff mode at a constant coronary perfusion pressure of 20 mm Hg as described in Chapter 2. The hearts were perfused with Krebs-Henseleit solution containing 11 mM glucose, 0.4 mM [1-^{14}C]palmitate, 3% bovine serum albumin, with or without 100 μU/ml insulin. Fatty acid oxidation rates were measured by quantitative collection of $^{14}CO_2$ every 10 min as described in Section 2.3.1. Plasma concentration of the full-length adiponectin was 6.8 ± 1.8 μg/ml in 7-day-old rabbits (see *Section 5.3.1. Plasma Levels of Adiponectin in 1-day, 7-day, and 6-week-old Hearts*). Therefore, adiponectin was added at a concentration of 10 μg/ml. The amount of gAd (1.5 μg/ml) in the perfusate was based on the calculation of equimolar concentration of the truncated peptide (i.e. the concentration of both the full-length adiponectin, and the gAd was ~100 nM).

Tissue Preparation for AMPK and ACC Activity Assays

Approximately 50 mg frozen ventricular tissue from previously perfused hearts were homogenized for AMPK and ACC activity assays as described in Section 2.4.2.

AMPK Activity Assay

AMPK activity assay was performed as described in Chapter 2.

ACC Activity Assay

ACC activity assay was performed as described in Chapter 2.

Western Blot Analysis of Ser79-phosphorylated ACC

Heart homogenates (30 μg protein) were subjected to SDS-polyacrylamide gel electrophoresis as described in Chapter 2. Following the gel electrophoresis, the protein bands were transferred to nitrocellulose. Membranes were then probed with rabbit polyclonal antibody against phosphorylated (Ser79) ACC (Upstate Biotechnology, 1:2000 dilution in Tris-buffered saline with 0.05% Tween 20 in 1% non-fat milk). Secondary peroxidase-conjugated goat anti-rabbit IgG was used to visualize phospho-ACC. Chemiluminescent detection was performed on the membranes using an ECL Western Blot detection kit.

Determination of CoA Esters

CoA esters were determined on 6% perchloric acid extracts from frozen heart tissues using a modified HPLC procedure as described in Chapter 2.

5.3. Results

5.3.1. Plasma Levels of Adiponectin in 1-day, 7-day, and 6-week-old Hearts

Figure 5.1 shows the relative amounts of adiponectin in plasma samples collected from 1-day and 7-day-old rabbits. Figure 5.1A is a representative Western blot, and Figure 5.1B is the densitometric analysis of Figure 5.1A. Plasma levels of adiponectin increase between 1-day and 7-days of age. The antibody that we used (Chemicon) in this experiment was raised against the gAd domain of the peptide. We also observed a signal that corresponds to a ~16 kDa protein (gAd) on the immunoblots. However, the signal was very weak, and did not allow us to determine the amount of gAd in the plasma.

An example of the adiponectin standard curve that was used to determine the amount of plasma adiponectin was shown in Figure 5.2. As shown in Table 5.1, plasma concentrations of the full-length adiponectin were 1.2 ± 0.3 μg/ml in 1-day, 6.8 ± 1.8 μg/ml in 7-day, and 45 ± 5.0 μg/ml in 6-week old rabbits.

5.3.2. Fatty Acid Oxidation in 1-day-old Hearts in the Presence of 10 μU/ml Insulin

Fatty acid oxidation rates were measured in 1-day-old hearts in the presence of 10 μU/ml insulin. When added, adiponectin (10 μg/ml) was present throughout the entire 40 min perfusion period. The recombinant full-length adiponectin preparation at this concentration did not affect fatty acid oxidation rates in 1-day-old rabbit hearts. Cumulative fatty acid oxidation in control and adiponectin-treated hearts were shown in Figure 5.3A. Figure 5.3B shows the average fatty acid oxidation rates throughout the 40 min perfusion in control and adiponectin-treated hearts.

5.3.3. Fatty Acid Oxidation in 1-day-old Hearts in the Absence of Insulin

Figure 5.4 shows fatty acid oxidation rates in 1-day-old hearts perfused in the absence of insulin. In this of set experiments, gAd (1.5 μg/ml) was added at 30 min of the perfusion (Figure 5.4A). gAd resulted in a rapid increase in fatty acid oxidation compared to control hearts (Figure 5.4A). Comparison of the last 10 min of the perfusion to the initial 30 min period (Figure 5.4B) showed that gAd more than doubled fatty acid oxidation rates.

5.3.4. AMPK Activity of Control and gAd-treated Hearts in the Absence of Insulin

To determine whether AMPK pathway is involved for the effects of gAd on fatty acid oxidation, AMPK activity was measured at the end of the 40 min perfusion period in control and gAd-treated hearts. As shown in Figure 5.5, AMPK activity was similar between control and gAd-treated hearts.

5.3.5. Phosphorylation and Activity of ACC in Control and gAd-treated Hearts

To verify that the increase in fatty acid oxidation was indeed independent of AMPK pathway, the phosphorylation status of Ser79 and activity of ACC were examined (Ser79 is the AMPK phosphorylation site on ACC). Figure 5.6A is a western blot showing Ser79 phosphorylated ACC. As reported earlier by our group (Lopaschuk et al 1994), rabbit heart expresses both isoforms of ACC (ACC265 and ACC280) to an equal extent. gAd treatment did not alter the extent of ACC Ser79 phosphorylation (Figure 5.6A and 5.6B) nor the activity of ACC (Figure 5.6C).

5.3.6. Malonyl CoA and Acetyl CoA Levels

The presence of did not change the amount of malonyl CoA (Table 5.2). The amount of acetyl CoA tended to increase, however this increase did not reach statistical significance (Table 5.2).

5.3.7. Fatty Acid Oxidation in 1-day-old Hearts in the Presence of Insulin

To determine if insulin altered the gAd-stimulated increase in fatty acid oxidation, fatty acid oxidation rates were measured in the presence of 100 μU/ml insulin with or without 1.5 μg/ml gAd. In the first set, gAd was added at the beginning of the perfusion protocol. In the presence of 100 μU/ml insulin, fatty acid oxidation rates were similar in control and gAd-treated hearts throughout the 40 min perfusion (Figure 5.7A). Addition of 3 μg/ml gAd at the beginning of the protocol did not affect fatty acid oxidation rates in the presence of insulin (results not shown).

We extended our examination in these hearts further, and added gAd (1.5 μg/ml) during the last 10 min of the perfusion to provide similar conditions as in the non-insulin perfusion series. gAd had no effect on fatty acid oxidation in the presence of 100 μU/ml insulin (Figure 5.7B).

5.4. Discussion

The newborn period is characterized by dramatic alterations in energy substrate supply, with fatty acid oxidation becoming a predominant source of energy following birth (reviewed in Lopaschuk et al 1992). Alterations in hormone concentrations play a role in this process, with a decrease in insulin and increase in glucagon contributing to the increase in fatty acid oxidation (Makinde et al 1998). In this study we are showing that the plasma levels of adiponectin increase following birth and may contribute to the postnatal increase in fatty acid oxidation. In particular, a truncated form of adiponectin, gAd, was shown to acutely increase fatty acid oxidation in 1-day-old rabbit hearts. In contrast, full-length adiponectin did not have any effect on fatty acid oxidation rates. In previous studies, we have shown that a decrease in malonyl CoA levels contributes to the increase in fatty acid oxidation in the newborn heart (Lopaschuk et al 1994). Of interest, is that this effect of gAd on fatty acid oxidation occurred independent of changes in malonyl CoA levels, or AMPK and ACC control of malonyl CoA levels.

Adiponectin is a peptide hormone, and is secreted exclusively by the differentiated adipocytes (Scherer et al 1995). Although the initial signal to increase the plasma levels of adiponectin is currently not known, a decrease in insulin may be the triggering event that increases adiponectin gene expression. Insulin decreases adiponectin mRNA in a 3T3-L1 cell line in a dose-dependent fashion (Fasshauer et al 2002). Whether the suppressant effect of insulin on mRNA is translated to the protein was not addressed in that study. In this study, we show that plasma adiponectin levels dramatically increase following birth, at the same time insulin levels decrease (Girard et al 1992).

Recently, Combs et al (2003) also showed that levels of adiponectin increase in postnatal life in mice. The time course of the maturation of mouse heart is not known. However, we have previously shown that in rabbit heart fatty acid oxidation increases within the first week of the postnatal life. This is consistent with the rise in adiponectin between 1-day and 7-day following birth (Figure 5.1).

Adiponectin is present in serum at high concentrations and exists as several molecular weight forms in the plasma (Scherer et al 1995). This raises questions as to the precise composition in the plasma, and more importantly, what is the active form of this protein. Recently, Tsao et al (2002) reported that the largest species (apparent molecular mass, 410 kDa) produced by E. Coli was an adiponectin hexamer. However, adiponectin also forms high molecular weight (HMW) species of apparent molecular weight ~629 kDa in the plasma of both mice and human (Tsao et al 2002, Pajvani et al 2003). In addition to full-length adiponectin and its higher molecular weight complexes, it has also been shown that the truncated form of adiponectin is biologically active. In particular, the globular domain of adiponectin, gAd, has been shown to stimulate fatty acid oxidation in muscle (Fruebis et al 2001). Unfortunately, we were unable to determine the amount of gAd in the plasma with the antibody used, however, gAd was found to be a potent stimulator of fatty acid oxidation in the newborn heart.

During the immediate postnatal period plasma glucagon increases and plasma insulin falls and remains in a low range concentration in newborns of different species (reviewed in Girard et al 1992). It has been suggested that the neonatal increase in plasma glucagon and the fall in plasma insulin could be related to the stress of birth through an activation of the sympathetic nervous system (Girard et al 1973, Sperling et al 1984). Catecholamines may trigger changes in plasma insulin and glucagon during the immediate newborn period, since they are potent stimuli of glucagon release and inhibitors of insulin release (Eliot et al 1980, Padbury et al 1988). Previously, it was reported that both the expression and activity of AMPK increases between 1-day and 7-days in newborn rabbit hearts (Makinde et al 1997). It was also shown that insulin inhibits AMPK activity in the heart (Gamble and Lopaschuk 1997), suggesting that a decrease in insulin levels *in vivo* may stimulate AMPK activity following birth (Makinde et al 1997). As a result, insulin may have a dual role following birth, directly alters malonyl CoA and fatty acid oxidation in the newborn heart, and indirectly alters hormone levels such as adiponectin.

Two independent groups recently showed that adiponectin/gAd increases fatty acid oxidation by stimulating AMPK (Yamauchi et al 2002, Tomas et al 2002). AMPK phophorylates and inactivates ACC, the enzyme that is responsible for malonyl CoA synthesis. Since gAd stimulated fatty acid oxidation in the newborn heart, we investigated the involvement of AMPK-ACC-malonyl CoA pathway in gAd-induced increase in fatty acid oxidation. We did not observe a change in phosphorylation status or activity of ACC. Malonyl CoA levels were also similar between control and gAd-treated hearts. Our results indicated that the increase in fatty acid oxidation induced by gAd was independent of AMPK.

In our initial perfusions, we included insulin (10 μU/ml) to the perfusate. Addition of full-length adiponectin at a concentration similar to that seen in the plasma of 7-day-old rabbits did not affect the fatty acid oxidation rates in the presence of this concentration of insulin. The levels of plasma adiponectin that we report in this study (Table 5.1) are based on the trimeric structure that has a molecular weight of 90 kDa (3x 30 kDa). When selecting the concentration for treatment, we based our calculations on the trimeric form of adiponectin. We used a bacterial expression system (E. Coli) to prepare the full-length adiponectin. The protein mixture prepared by this system has been shown to contain the trimeric and hexameric forms of adiponectin, but not the high molecular weight (HMW) form (Tsao et al 2002, Berg et al 2002). Therefore, the lack of effect with adiponectin treatment in our perfusions may be the result of the absence of the HMW form of adiponectin in our preparation.

Two groups have shown that the stimulatory effect of gAd on fatty acid oxidation in skeletal muscle is mediated by AMPK activation (Yamauchi et al 2002, Tomas et al 2002). We therefore examined gAd activation of AMPK activity stimulated fatty acid oxidation. When added at the last 10-min of the perfusion, gAd increased fatty acid oxidation rates compared to the control hearts. As shown in Figure 5.5 and Figure 5.6, gAd-induced increases in fatty acid oxidation were not mediated by AMPK-ACC-malonyl CoA pathway. The mechanism by which gAd increased fatty acid oxidation remains to be determined. Of interest is that insulin (100 μU/ml) could overcome the gAd stimulation of fatty acid oxidation. This suggests that the mechanism by which gAd stimulates fatty acid oxidation involves a pathway that can be inhibited by insulin.

While this thesis was in preparation, Yamauchi et al (2003) cloned complementary DNAs encoding adiponectin receptors AdipoR1 and AdipoR2. The authors showed that AdipoR1 is abundantly expressed in skeletal muscle and AdipoR2 is the predominant form in the liver. They also suggested that AdipoR1 is a high-affinity receptor for gAd and a very low-affinity receptor for full-length adiponectin. The lack of effect with full-length adiponectin (Figure 5.3) may be the result of the presence of AdipoR1 in the heart. Expression of AdipoR1 in C2C12 myocytes increased fatty acid oxidation and glucose uptake on stimulation with gAd, and these effects were only partially inhibited by dominant negative-AMPK or SB203580, a specific inhibitor of p38 MAPK. Therefore, the authors suggested that other signaling pathways are involved in the biological effects of adiponectin and gAd .

In conclusion, our results suggest that a decrease in plasma insulin and increase in gAd are involved in the increase of cardiac fatty acid oxidation in the immediate newborn period. The increase in fatty acid oxidation is not mediated by AMPK-ACC-malonyl CoA pathway.

On the information provided here, we propose the scheme shown in Figure 5.8. The identification of the signal that results in the cleavage of the full-length adiponectin to gAd, and the reduction of the HMW pool of adiponectin to lower molecular forms are required to map the intracellular events that result in the maturation of fatty acid oxidation in the newborn period.

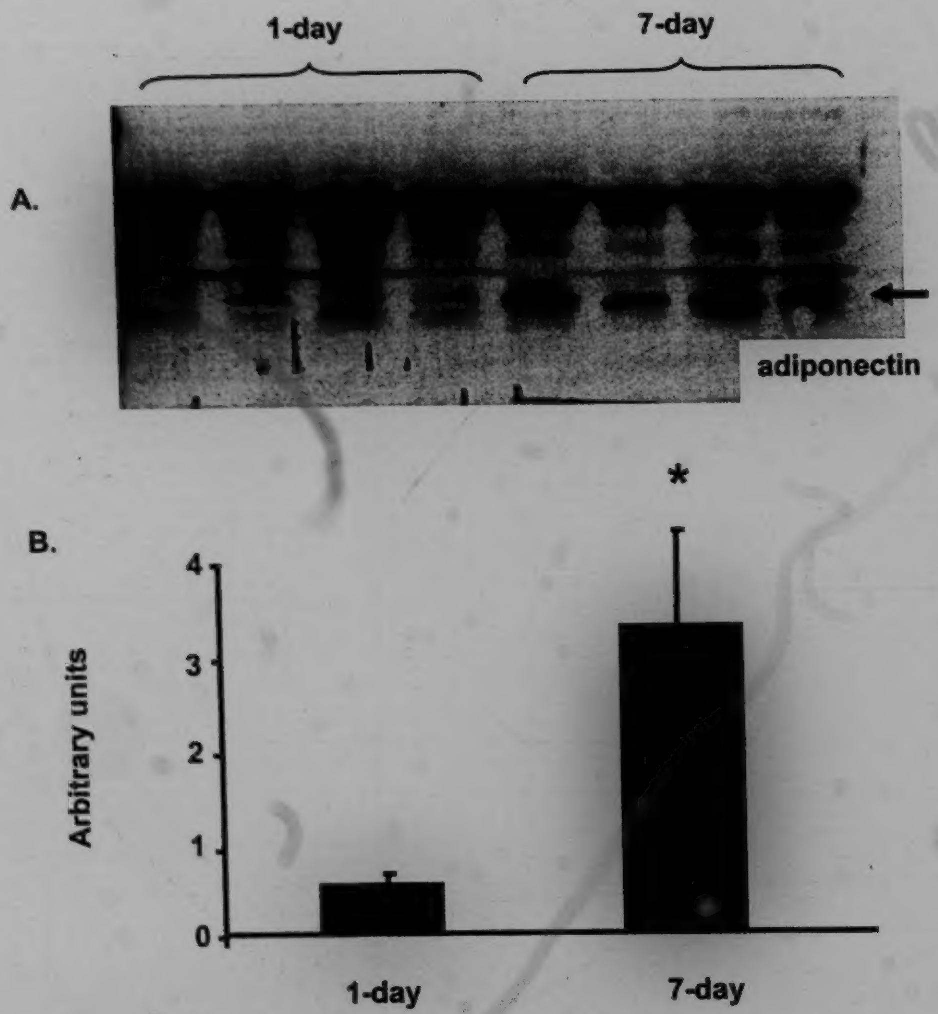


Figure 5.1. The relative amounts of full-length adiponectin in the plasma samples from 1-day and 7-day-old rabbits

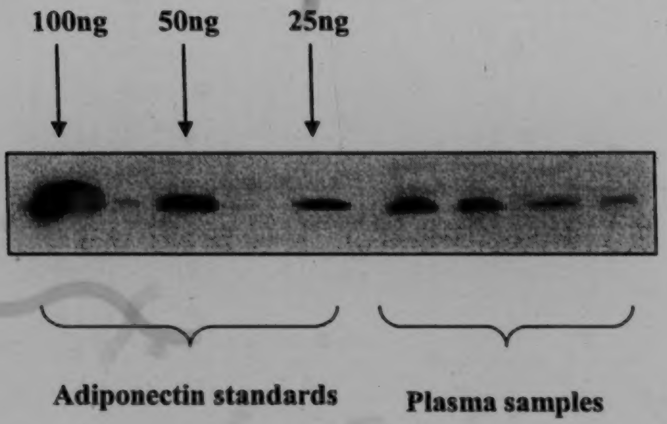
A. Representative Western blot showing the relative amounts of the full-length form of adiponectin in plasma samples from 1-day and 7-day-old hearts. Each lane represents an individual rabbit.

B. Densitometric analysis of the data in Figure 5.1A. Values in the bar graph are mean ± S.E. of 4 plasma samples in each age group.

Figure 5.2. Quantitative Western blot for the measurement of plasma adiponectin levels

A. Representative immunoblot showing increasing amounts of recombinant adiponectin and plasma samples from 1-day and 7-day-old rabbits.

B. Standard curve for recombinant adiponectin. The units on y-access are the results of densitometric analysis of the blots on Figure 5.2A. The densities of the blots that correspond to the plasma samples were fitted to the curve after the confirmation of the linearity of the standard curve.


100ng
50ng
25ng
Adiponectin standards
Plasma samples

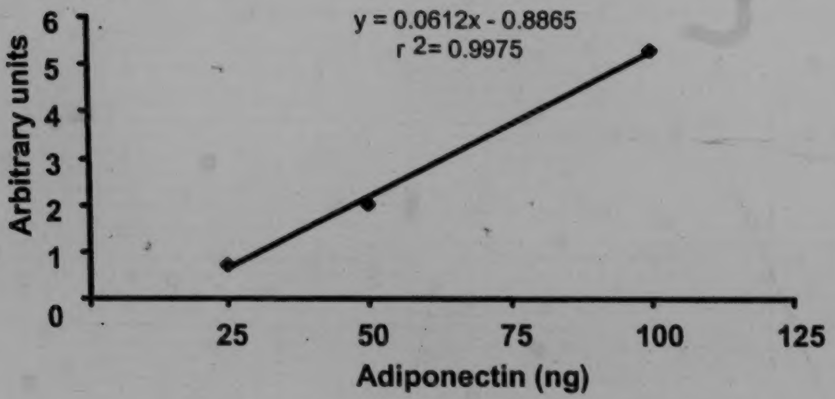

y = 0.0612x - 0.8865
r 2= 0.9975
Arbitrary units
0
1
2
3
4
5
6
25
50
75
100
125
Adiponectin (ng)

Table 5.1. The levels of adiponectin in newborn rabbit plasma

Age	1-day	7-day	6-week
Adiponectin (μg/ml)	1.2 ± 0.3	6.8 ± 1.8	45 ± 5.0

Plasma levels of full-length adiponectin were determined using a quantitative Western blotting technique as described in detail in the "Experimental Procedures" section.

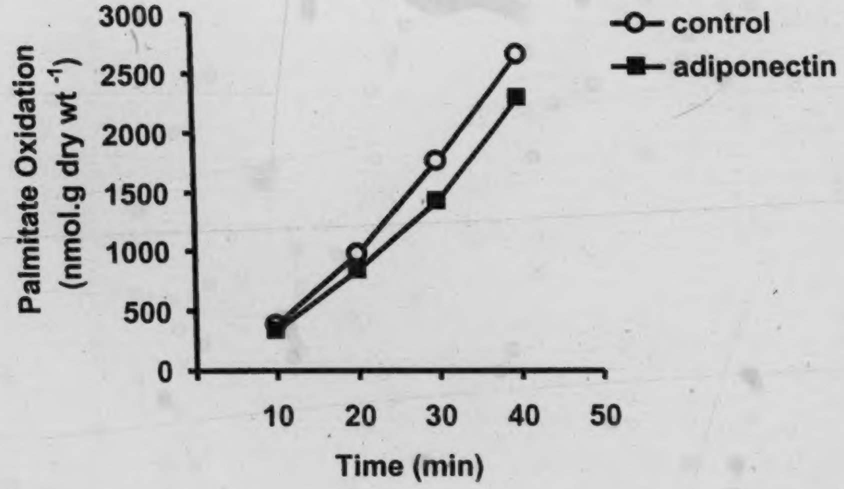
Values are mean ± S.E. of 4 rabbits in each group.

Figure 5. 3. Effect of adiponectin on palmitate oxidation rates in 1-day-old hearts perfused in the presence of 10 μU/ml insulin

Cumulative (A) and steady state (B) palmitate oxidation rates were measured in 1-day-old hearts using [1-^{14}C]palmitate as described in "Experimental Procedures". Adiponectin (10 μg/ml) was added at the beginning of the 40 min perfusion protocol.

Values are mean ± S.E. of 6 control and 7 adiponectin-treated hearts.

A.


Palmitate Oxidation
(nmol.g dry wt $^{-1}$)
3000
2500
2000
1500
1000
500
0
10
20
30
40
50
Time (min)
-○- control
-■- adiponectin

B.

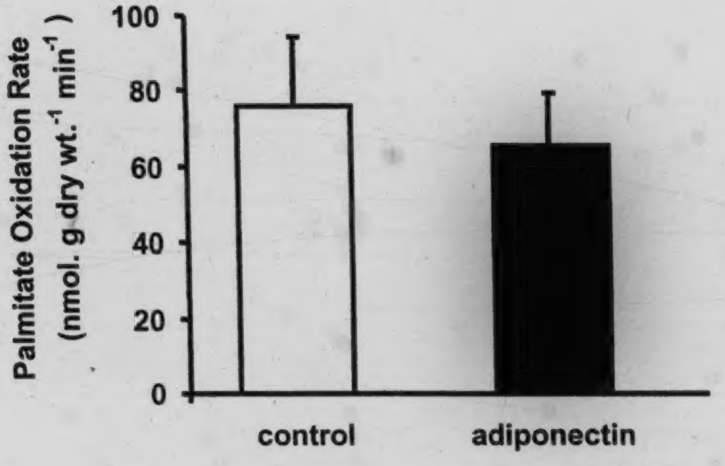
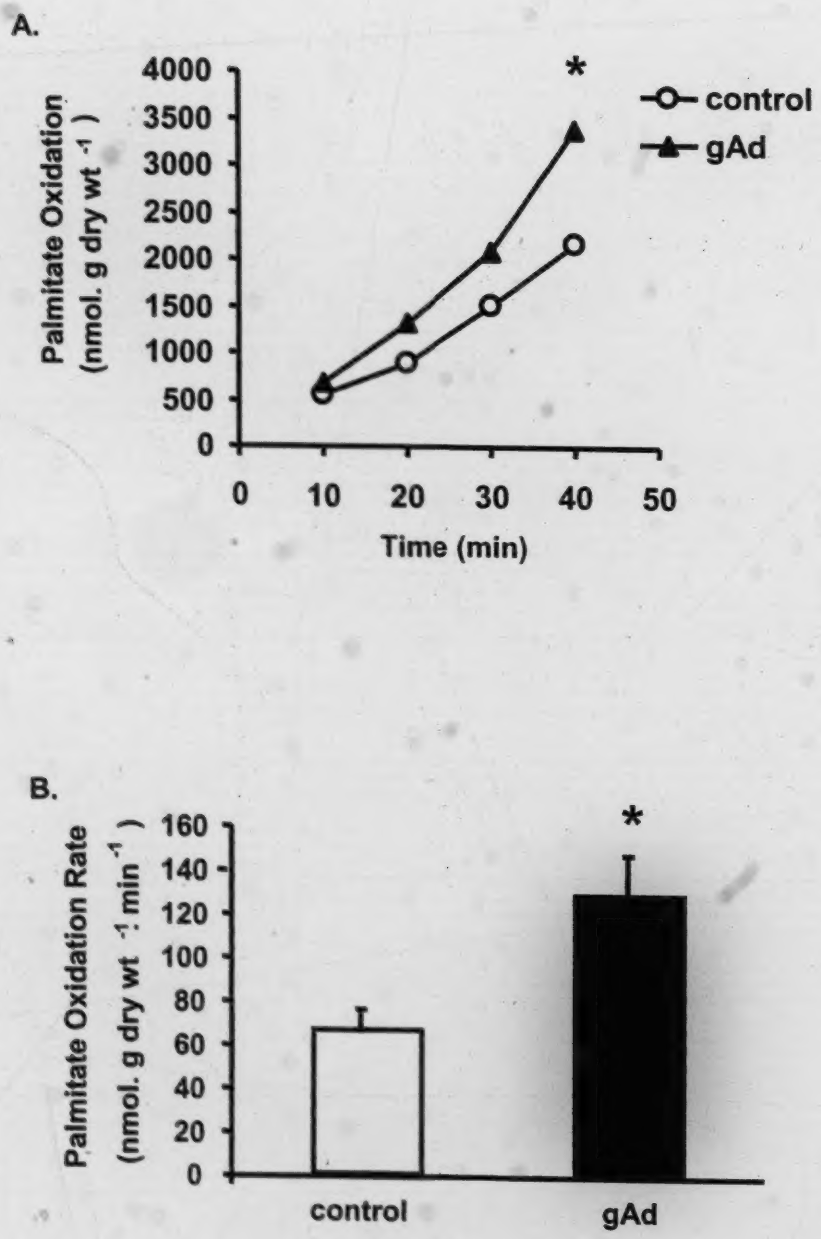

Palmitate Oxidation Rate
(nmol. g dry wt.$^{-1}$ min^{-1})
100
80
60
40
20
0
control
adiponectin

Figure 5.4. Effects of gAd on palmitate oxidation rates in 1-day-old hearts perfused in the absence of insulin

Cumulative (A) and steady state (B) palmitate oxidation rates were measured in 1-day-old hearts using [1-^{14}C]palmitate as described in "Experimental Procedures". The total length of perfusion was 40 min. gAd (1.5μg/ml) was present during the last 10 min of the perfusion protocol.

Values are mean ± S.E. of 7 hearts in each group.

* significantly different from palmitate oxidation rate in the control group.


A.
Palmitate Oxidation
(nmol. g dry wt -1)
4000
3500
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
50
Time (min)
*
control
gAd
B.
Palmitate Oxidation Rate
(nmol. g dry wt -1 . min -1)
160
140
120
100
80
60
40
20
0
*
control
gAd

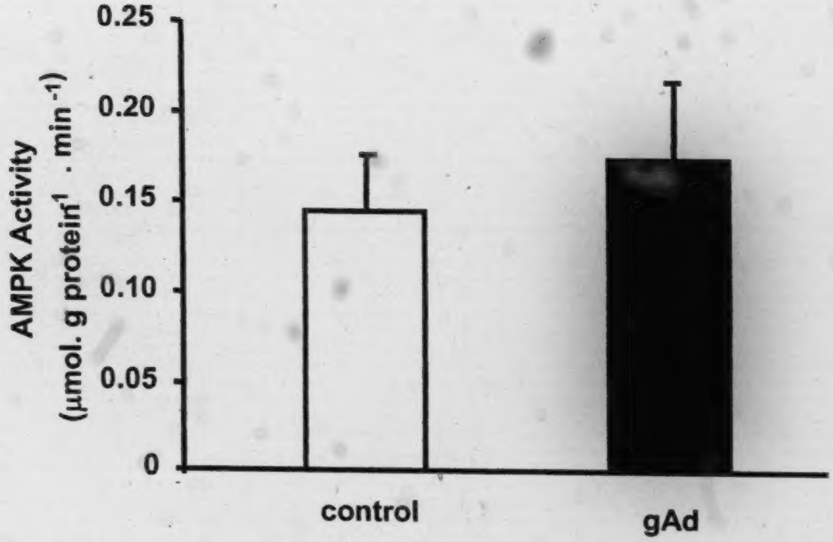


Figure 5.5. AMPK activity in 1-day-old rabbit hearts perfused with or without gAd

AMPK activity was measured in 1-day-old hearts perfused with (gAd) or without (control) gAd in the last 10 min of the perfusion protocol as described in the "Experimental Procedures" section. The perfusate did not include insulin. Values are mean ± S.E. of 7 hearts in each group.

Figure 5.6. Effects of gAd on Ser79-phosphorylation and ACC activity in 1-day-old rabbit hearts

A. Immunoblot analysis of Ser79-phosphorylated ACC in 1-day-old hearts perfused with (gAd) or without (control) gAd. The perfusate did not include insulin.

Each lane represents one heart.

B. Densitometric analysis of the immunoblot shown in Figure 5A. Values in the bar graph are mean ± S.E.

C. ACC activity was measured in 1-day-old hearts perfused with (gAd) or without (control) gAd.

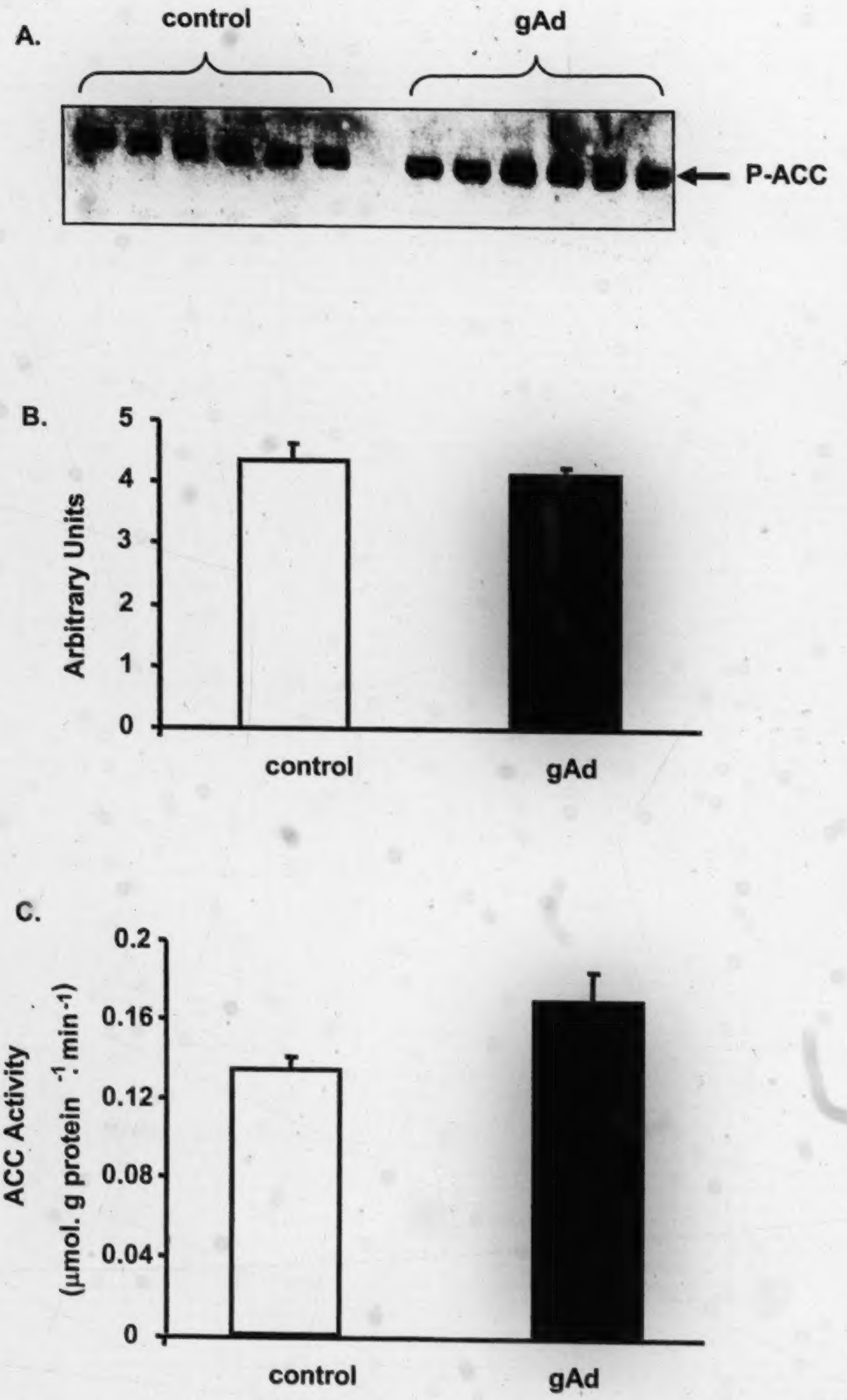

A.
control
gAd
P-ACC
B.
Arbitrary Units
0
1
2
3
4
5
control
gAd
C.
ACC Activity
(µmol. g protein -1. min-1)
0
0.04
0.08
0.12
0.16
0.2
control
gAd

Table 5.2. Malonyl CoA and Acetyl CoA Contents in 1-day-old hearts

	control	**gAd**
Malonyl CoA *(pmol.mg dry wt^{-1})*	2.3 ± 0.2	2.3 ± 0.4
Acetyl CoA *(pmol.mg dry wt^{-1})*	85.8 ± 3.9	121.6 ± 26.7

Malonyl CoA and acetyl CoA levels were measured at the end of the 40 min perfusion in control hearts, and in the hearts that had been treated with 1.5 μg/ml gAd in the last 10 min of the perfusion protocol.

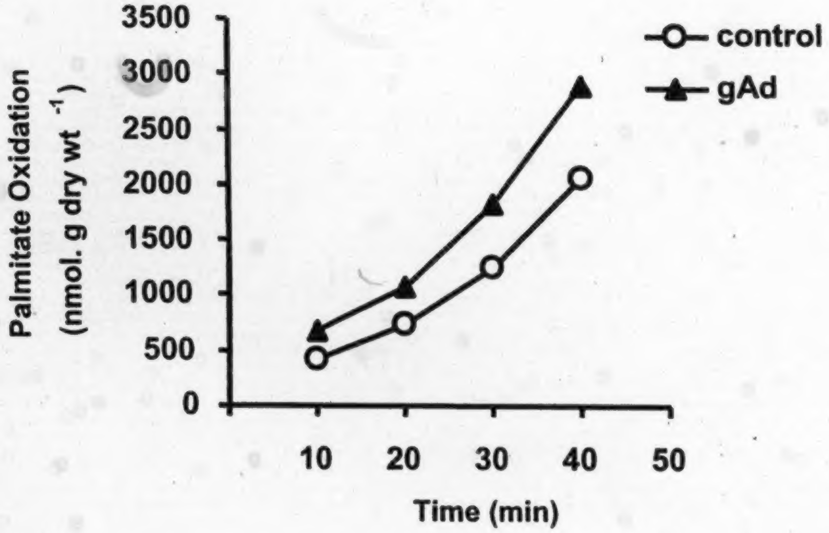
Values are mean ± S.E. of 7 hearts in each group.

Figure 5.7. Effects of gAd on palmitate oxidation rates in 1-day-old hearts perfused in the presence of insulin

Cumulative (A) and steady state (B) palmitate oxidation rates were measured in 1-day-old hearts in the presence of (100 μU/ml) insulin using [1-^{14}C]palmitate as described in "Experimental Procedures". gAd (1.5μg/ml) was during the last 10 min of the perfusion protocol.

Values are mean ± S.E. of 5-7 hearts in all groups.

A.


Palmitate Oxidation
(nmol. g dry wt $^{-1}$)
3500
3000
2500
2000
1500
1000
500
0
10
20
30
40
50
Time (min)
control
gAd

B.

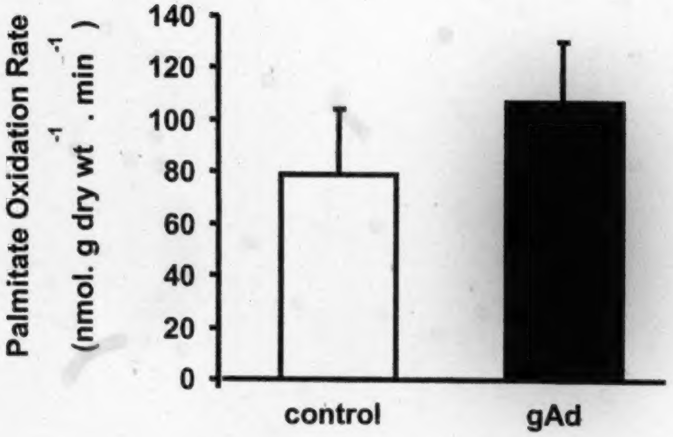
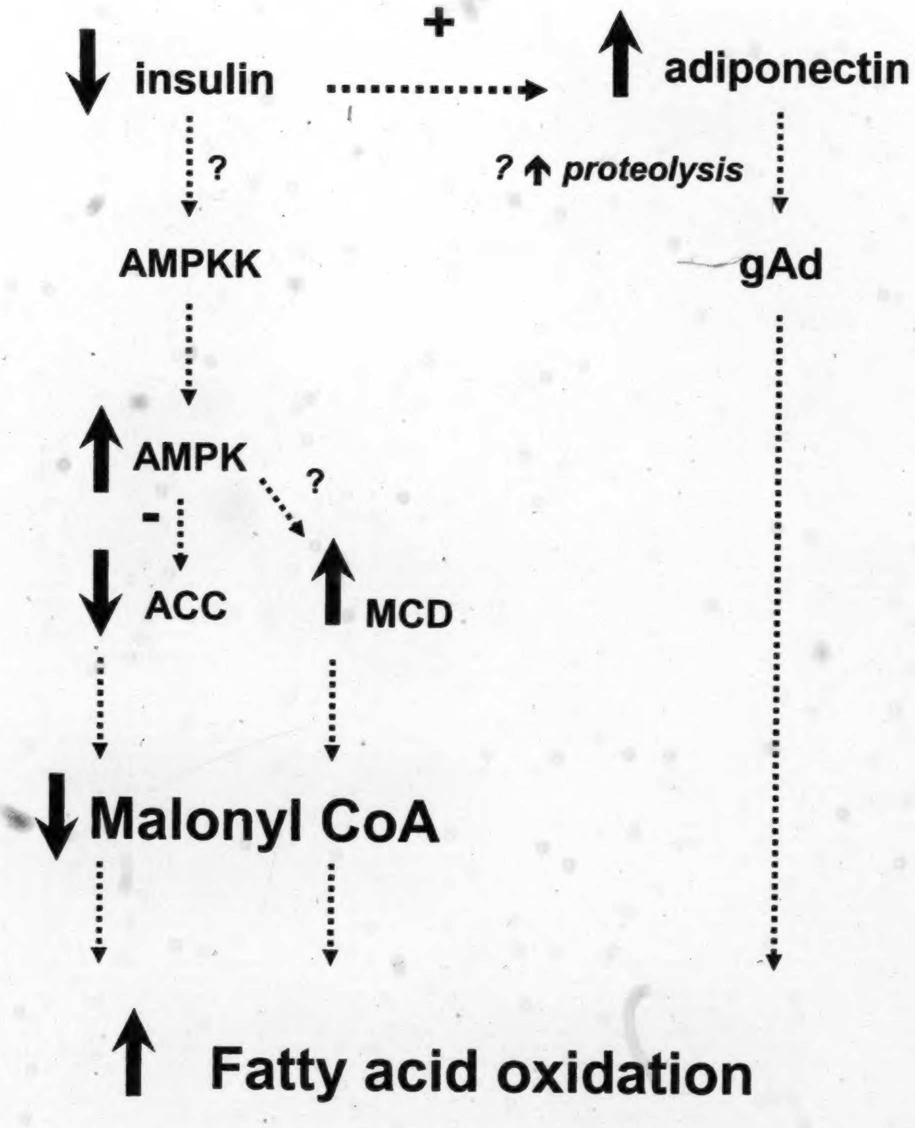

Palmitate Oxidation Rate
(nmol. g dry wt $^{-1}$. min $^{-1}$)
140
120
100
80
60
40
20
0
control
gAd

Figure 5.8. Proposed pathway by which fatty acid oxidation increases in the newborn heart

Plasma insulin decreases as a result of sympathetic activation and an increase in catecholamine release. A decrease in insulin relieves the inhibition of AMPK resulting in an increase in AMPK activity. The increase in both AMPK expression and activity results in a decrease in ACC activity. MCD expression and activity also increases. A decrease in ACC activity and increase in MCD activity in the newborn period results in a dramatic drop in malonyl CoA. The decrease in insulin triggers adiponectin/gAd release from the adipocytes. Adiponectin is cleaved to gAd which stimulates fatty acid oxidation. This occurs via an AMPK-ACC independent pathway.


+
insulin
adiponectin
?
? proteolysis
AMPKK
gAd
AMPK
?
-
ACC
MCD
Malonyl CoA
Fatty acid oxidation

References

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Chapter 6

gAd-Globular Head Domain of Adiponectin Protects the Ischemic Heart by Stimulating Glucose Oxidation in Newborn Rabbits

This study was presented at Scientific Sessions 2002 of American Heart Association as an oral presentation and the abstract was selected for "Basic Research Trainee Award".

A paper is presently being prepared for submission to "Journal of Pediatric Research":
Besikci, A.O., Altarejos, J., Lopaschuk, G.D. *gAd-Globular Head Domain of Adiponectin Protects the Ischemic Heart by Stimulating Glucose Oxidation in Newborn Rabbits*

Cloning of the cDNA in pET-30a and optimization of the FPLC procedure were carried out by Ms. Judith Altarejos.

6.1. Introduction

Adiponectin is a polypeptide of ~30-kDa molecular weight. It has a signal sequence at the amino terminus, a small nonhelical region, a stretch of repeated collagens and a C-terminal globular head domain (gAd) that makes up for the majority of the protein (reviewed in Nakano et al 1999). The C-terminal complement factor C1q-like globular domain is a characteristic feature of a group of proteins known as collagen superfamily (Kishore and Reid 1999). Protein cleavage is a common feature of this family of proteins. Indeed, a proteolytic cleavage product of adiponectin increases fatty acid oxidation in muscle and causes weight loss in mice (Fruebis et al 2001). Recently, Yamauchi et al (2002) demonstrated that gAd and full-length adiponectin stimulated 5'-AMP-activated protein kinase (AMPK) in skeletal muscle. The authors showed that this activation increased the phosphorylation of acetyl CoA carboxylase (ACC), fatty acid oxidation, glucose uptake and lactate production in C2C12 myocytes. When administrated in vivo, both full-length adiponectin and gAd stimulated AMPK and increased the phosphorylation of ACC in mouse soleus muscle (Yamauchi et al 2002). Moreover, the same authors showed that stimulation of AMPK with full-length adiponectin reduced gluconeogenesis in the liver and plasma glucose in the same study. In agreement with these findings, Tomas et al (2002) recently reported that gAd stimulates fatty acid oxidation in skeletal muscle by activating AMPK and inactivating ACC.

Fatty acid oxidation in the newborn heart matures rapidly following birth (Werner et al 1989, Lopaschuk and Spafford 1990). For example, in 1-day old rabbit hearts, fatty acid oxidation rates are very low, and contribute less than 10% of the overall ATP production (Lopaschuk and Spafford 1990). However, by 7-days of age fatty acid oxidation dramatically increases, and becomes the predominant source of energy production. Our studies in 1-day-old rabbit hearts suggest that a decrease in plasma insulin and increase in gAd are involved in the increase of cardiac fatty acid oxidation in the immediate newborn period. The increase in fatty acid oxidation was not mediated by AMPK-ACC-malonyl CoA pathway (Chapter 5).

Previous studies suggest that the overall increase in energy-producing pathways improve the post-ischemic recovery in the newborn heart (Itoi and Lopaschuk 1996). In this study, we determined what effect gAd has on glucose and fatty acid oxidation in the newborn heart, as well as whether gAd alters the ability of the newborn heart to recovery following a severe ischemic insult.

6.2. Experimental Procedures

Animals

7-day-old New Zealand White rabbits were used in this study. When the rabbits totally lacked sensation, the thoracic cavity was opened and the heart quickly excised and placed in ice-cold Krebs-Henseleit solution. Blood was collected at the same time and plasma was separated by centrifugation (10,000 x g for 10 min).

Recombinant Protein Production and Protein Characterization

Recombinant adiponectin and gAd were produced by following the protocol described in Section 2.8.

Heart Perfusions

Isolated hearts obtained from 7-day-old hearts were perfused in the classical working heart mode, in which perfusate enters the left atrium and is injected into the aorta via the left ventricle as described in Section 2.2.2. All hearts were perfused with Krebs-Henseleit solution containing 3% bovine serum albumin, [9,10-^{3}H]palmitate, and 11 mM [U-^{14}C] or [5-^{3}H]glucose, and 100μU/ml insulin. To measure glycolysis, [5-^{3}H]glucose was added to the perfusate in a separate series of perfusions.

In one series, hearts were subjected to a 40 min aerobic perfusion. In the second series, hearts were perfused for an initial 30 min, following which a 40 min global ischemia was introduced. This was followed by a 40 min reperfusion period. Measurement of glucose oxidation, palmitate oxidation, and glycolysis was described in detail in Section 2.3.2. All these metabolic parameters were measured during the initial aerobic perfusion, and during the reperfusion period after ischemia.

Tissue Preparation for the AMPK and ACC Activity Assays

Approximately 50 mg frozen ventricular tissue obtained at the end of reperfusion was used for the enzyme activity assays. This procedure is described in Section 2.4.2.

AMPK and ACC Activity Assays

The enzyme activity assays were performed as described in Section 2.5.

6.3. Results

6.3.1. Plasma Levels of Adiponectin in 7-day-old Hearts

Plasma levels of adiponectin were determined in the plasma of 1-day, 7-day, and 6-week-old rabbits using a quantitative Western blotting technique as described in Chapter 5. The amount of the adiponectin was 6.8 ± 1.8 μg/ml in 7-day old rabbits (Chapter 5).

6.3.2. Mechanical Function of 7-day-old Hearts in the Presence and Absence of Adiponectin

The parameters related to the mechanical function of 7-day-old hearts are shown in Table 6.1. Heart rate was similar between the control and gAd-perfused hearts. However, peak systolic pressure and cardiac output increased in the gAd-perfused group compared to the control group.

6.3.3. The Rates of Fatty Acid Oxidation, Glucose Oxidation, and Glycolysis in 7-day-old Rabbit Hearts in the Presence and Absence of gAd

Previous studies have shown that fatty acid oxidation rates dramatically increase by 7-days of age (Lopaschuk et al 1991). The effect of gAd on fatty acid oxidation rates in 7-day-old rabbit hearts is shown in Figure 6.1A. gAd (1.5μg/ml) did not affect palmitate oxidation rates in 7-day-old rabbit hearts.

Of interest is that the rates of both glycolysis (Figure 6.1B) and glucose oxidation (Figure 6.1C) were increased in gAd-perfused hearts compared to control hearts.

The contribution of glucose oxidation and palmitate oxidation to overall TCA cycle acetyl CoA production is shown in Figure 6.2A. gAd increased the contribution of glucose oxidation to Krebs' Cycle acetyl CoA production from 15 ± 3% in control to 32 ± 4% in gAd-treated hearts.

Steady-state rates of ATP production are shown in Figure 6.2B. gAd increased total ATP production. Also, the contribution of glucose (glycolysis + glucose oxidation) to overall ATP production increased from ~21% in control hearts to 38% in gAd-treated hearts.

6.3.4. AMPK and ACC Activity in the Presence and Absence of gAd

AMPK activity was measured by the end of 40-min perfusions. AMPK activity was similar in control and gAd-treated hearts as shown in Figure 6.3.

We next measured ACC activity in control, and gAd-treated hearts by the end of 40-min perfusions. gAd, significantly increased the ACC activity as shown in Figure 6.4.

6.3.5. gAd in the Setting of Ischemia-reperfusion

Stimulation of glucose oxidation has been shown to improve the recovery of the heart after ischemia in the adult heart (Broderick et al 1993, McVeigh and Lopaschuk 1990). We next examined the effect of gAd on the recovery of contractile function and metabolism in 7-day-old hearts subjected to ischemia and reperfusion. As shown in Figure 6.5, during reperfusion, cardiac work recovered to ~50% in control hearts. The recovery of cardiac function was ~80% in gAd-treated hearts. The rates of glucose and fatty acid oxidation in aerobic perfusion and reperfusion periods in control and gAd-treated hearts are shown in Table 6.2. The rates of glucose oxidation were similar between control and gAd-treated hearts in both aerobic perfusion and reperfusion period (401 ± 54 and 617 ± 103 nmol.g dry wt^{-1}.min^{-1} in aerobic perfusion; 458 ± 72 and 517 ± 91 nmol.g dry wt^{-1}. min^{-1} in reperfusion in control and gAd-treated hearts, respectively). The rates of palmitate oxidation were also similar between control and gAd-treated hearts in both aerobic perfusion and reperfusion period (409 ± 73 and 505 ± 35 nmol.g dry wt^{-1}.min^{-1} in aerobic perfusion; 283 ± 82 and 271 ± 61 nmol.g dry wt^{-1}.min^{-1} in reperfusion in control and gAd-treated hearts, respectively).

6.4. Discussion

In this study we examined what effect gAd has on glucose and fatty acid metabolism in 7-day-old hearts. Our results suggested that gAd increases contractile function in newborn rabbit hearts. This increase is associated with an increase in glucose oxidation rates.

Previously, our laboratory reported that the increase in myocardial ATP production required to sustain contractile function following the administration of a positive inotropic agent occurs preferentially through an increase in glucose metabolism (Collins-Nakai et al 1994). Our experiments did not allow us to determine the exact cause-effect relationship between the stimulation of glucose oxidation and increased demand of the cardiac muscle.

Our study highlights the difference between the adult and the newborn heart in the setting of ischemia and reperfusion. Some of the metabolic differences between adult and newborn heart have been addressed previously by our laboratory. For example, adult and immature hearts differ in their responsiveness to the stimulation of PDC activity. In adult hearts, pharmacological stimulation of PDC by dichloroacetate (DCA) is associated with a significant improvement of mechanical recovery following ischemia (Lopaschuk et al 1993). However, the effects of DCA on functional recovery of immature hearts are less dramatic as shown by Itoi et al (1993) and Itoi and Lopaschuk (1996). The effects of Ca^{+2} in the immature heart also differ dramatically from what has been observed in mature hearts. In adult rat hearts, high Ca^{+2} concentrations were reported to be detrimental (Kuroda et al 1986, Broderick et al 1993). In the immature hearts, however, increase in extracellular Ca^{+2} resulted in an increase in the post-ischemic recovery (Itoi and Lopaschuk 1996). There is one common pathway in the aforementioned studies and our study: an overall increase in oxidative metabolism. In the study by Itoi and Lopaschuk (1996), an overall increase in palmitate, lactate, and glucose oxidation has been demonstrated when extracellular Ca^{+2} was increased. Ca^{+2} has also been shown to increase mitochondrial volume and stimulate palmitate oxidation in isolated heart mitochondria (Halestrap et al 1987). Similarly, we calculated the total ATP production using the metabolic rates from perfusion experiments and observed an increase in ATP production in gAd-treated hearts compared to control hearts. Similar results were observed when 7-day-old rabbit hearts were exposed to increased fatty acid supply (2.4 mM palmitate) in the setting of ischemia-reperfusion (Dr. Ivan Rebeyka, unpublished observations).

Increases in glucose uptake in C2C12 myocytes with gAd via stimulation of AMPK has been documented recently (Yamauchi et al 2002). We therefore measured the activities of AMPK and ACC in control, and gAd-perfused hearts. AMPK activity was similar between the two groups. However, gAd-perfused hearts had higher ACC activity compared to control hearts. Malonyl CoA in the heart is produced by the carboxylation of acetyl CoA by ACC. Two major isoforms of ACC have been identified, a 265 kDa (ACC265) and a 280 kDa (ACC280) isoform. Both isoforms are expressed in the heart (Abu-Elheiga et al 1997, Lopaschuk et al 1994). Acute regulation of ACC280 activity differs from ACC265. Although phosphorylation and inactivation by AMPK and PKA are important in the regulation of ACC265 (discussed in Chapter 1), ACC280 was suggested to be regulated primarily by substrate supply (Saddik et al 1993). Therefore, a higher activity in gAd-treated hearts might not be a direct indication of a higher phosphorylation status in this group compared to the control group. Although highly speculative, ACC might be stimulated by gAd, and the production of malonyl CoA might be related to the availability of the acetyl CoA in the cytosol. If acetyl CoA in the mitochondria is higher than the requirement for the Krebs' Cycle activity, the excess will be transferred to the cytosol via carnitine acetyl transferase. In that case, cytosolic concentration of acetyl CoA will increase and will drive ACC to produce malonyl CoA and thereby inhibit fatty acid transport into the mitochondria. If, however, the concentration of mitochondrial acetyl CoA only meets the requirements of Krebs' Cycle activity, acetyl CoA will not be transferred to the cytosol.

In summary, we report that the presence of gAd increases glucose oxidation and increases cardiac work in isolated working 7-day-old rabbit hearts.

Table 6.1. Mechanical function in control and gAd-treated 7-day-old rabbit hearts

	Control	gAd
Heart Rate *(bpm)*	211 ± 2	215 ± 3
Peak Systolic Pressure *(mm Hg)*	55 ± 1	59 ± 1 *
Cardiac Output *(ml/min)*	37 ± 2	52 ± 1 *

Values are mean ± S.E. of 5 control and 13 gAd-treated hearts.

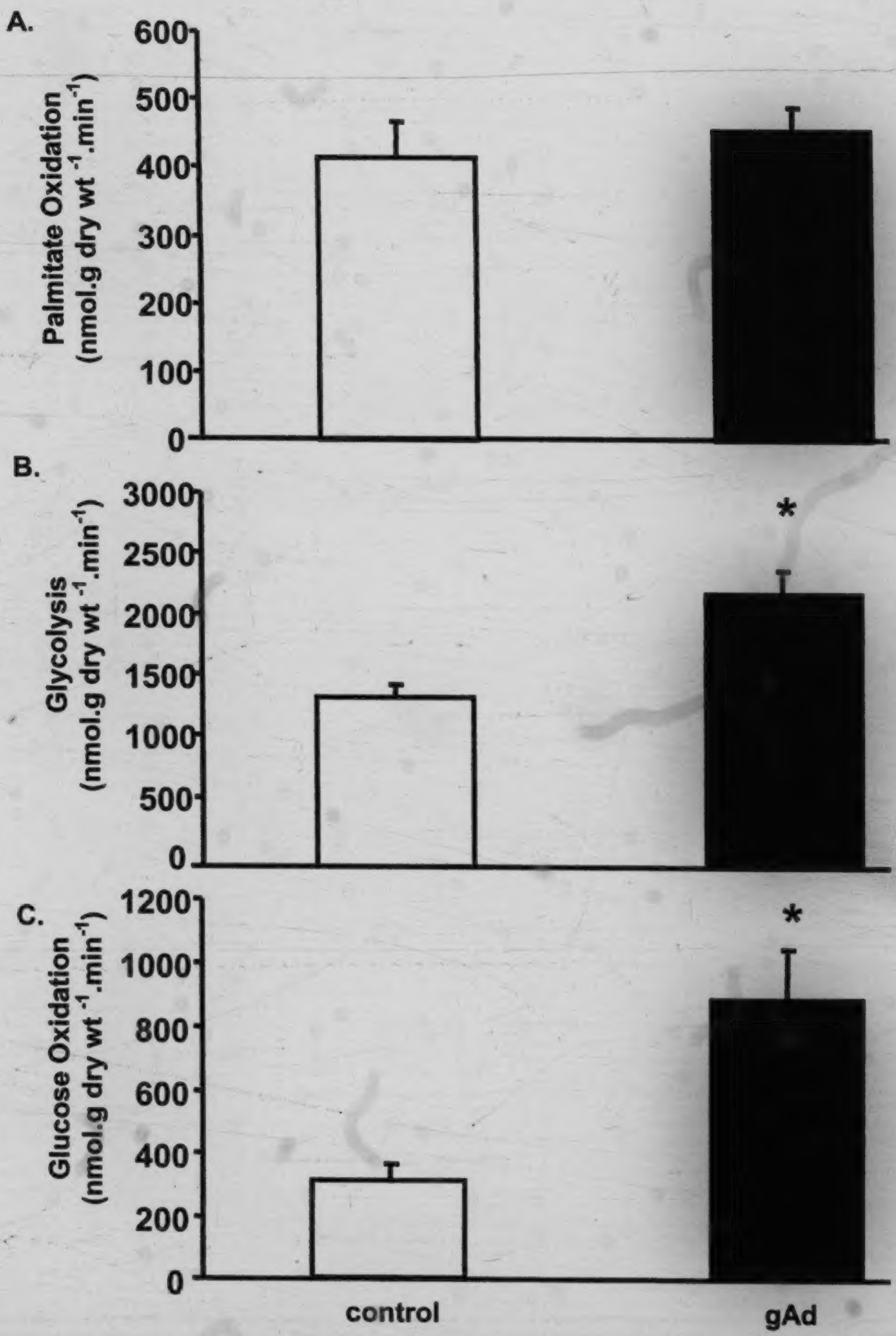
* significantly different from control hearts.

Figure 6.1. Effect of gAd on palmitate oxidation, glycolysis, and glucose oxidation in 7-day-old rabbit hearts

Steady state rates of fatty acid oxidation (A), glycolysis (B), and glucose oxidation (C) were measured in isolated working hearts as described in Chapter 2. Insulin was present at a concentration of 100 µU/ml in the perfusate. gAd (1.5 µg/ml) was added at the beginning of the 40 min perfusion protocol.

Values are mean ± S.E. of 5 control and 13 gAd-treated hearts.

* significantly different from control hearts.



A.
Palmitate Oxidation
(nmol.g dry wt $^{-1}$.min^{-1})
600
500
400
300
200
100
0
B.
Glycolysis
(nmol.g dry wt $^{-1}$.min^{-1})
3000
2500
2000
1500
1000
500
0
*
C.
Glucose Oxidation
(nmol.g dry wt $^{-1}$.min^{-1})
1200
1000
800
600
400
200
0
*
control
gAd

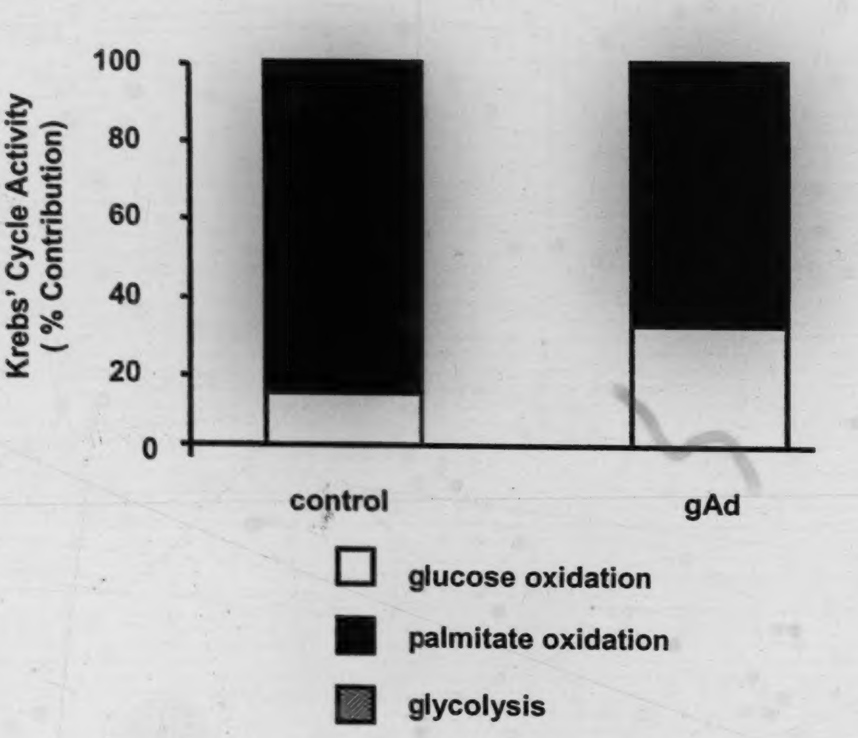
Figure 6.2. The effects of gAd on the contribution of fatty acid and glucose oxidation to overall acetyl CoA production (A) and ATP production (B) in 7-day-old rabbit hearts

The hearts were perfused with (gAd) or without (control) 1.5 μg/ml gAd.

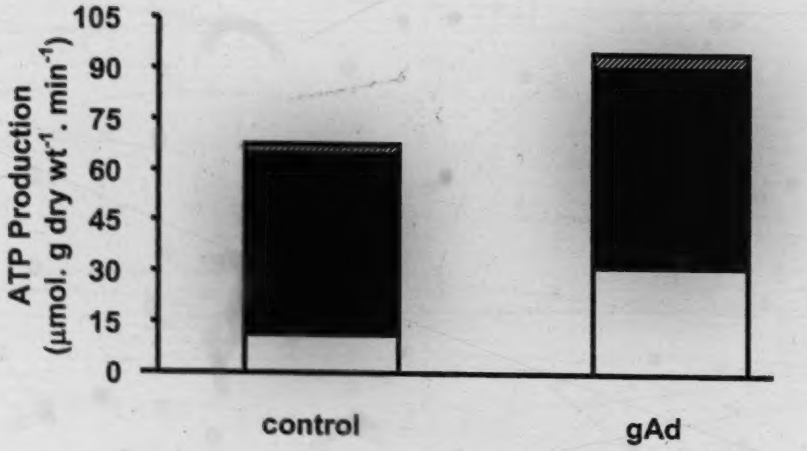
A. Acetyl CoA production by each pathway was calculated from fatty acid and glucose oxidation rates (Figure 6.1A, and Figure 6.1C, respectively), using a value of 2 moles of acetyl CoA produced per mole of glucose oxidized, and 8 moles of acetyl CoA per mole of palmitate oxidized.

B. ATP production by each pathway was calculated from fatty acid and glucose oxidation rates (Figure 6.1A, and Figure 6.1C, respectively), using a value of 129 moles of ATP produced per mole of palmitate oxidized, 2 moles of ATP produced per mole of glucose through glycolysis, and 36 moles ATP produced per mole of glucose oxidized.

A.


Krebs' Cycle Activity
(% Contribution)
100
80
60
40
20
0
control
gAd
☐ glucose oxidation
■ palmitate oxidation
▨ glycolysis

B.


ATP Production
(µmol. g dry wt^{-1}. min^{-1})
105
90
75
60
45
30
15
0
control
gAd

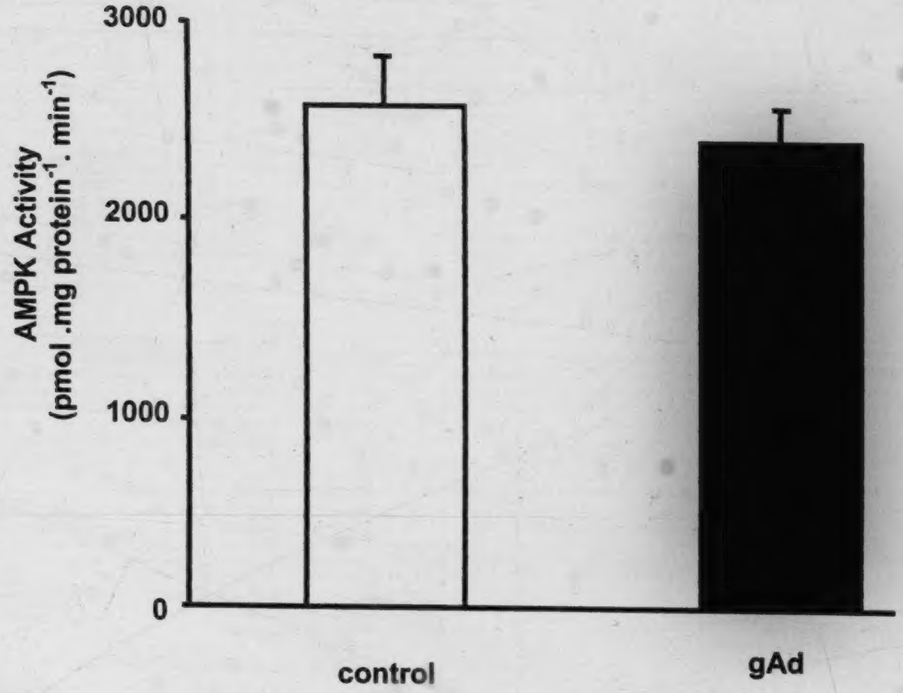


Figure 6.3. Effect of gAd on AMPK activity in 7-day-old working hearts

AMPK activity was measured in control and gAd-treated (1.5μg/ml) 7-day-old hearts as described in Chapter 2.

The activity of AMPK was determined in whole tissue homogenates of the hearts that were frozen at the end of 40 min perfusion.

Values are mean ± S.E. of 8 hearts in each group.

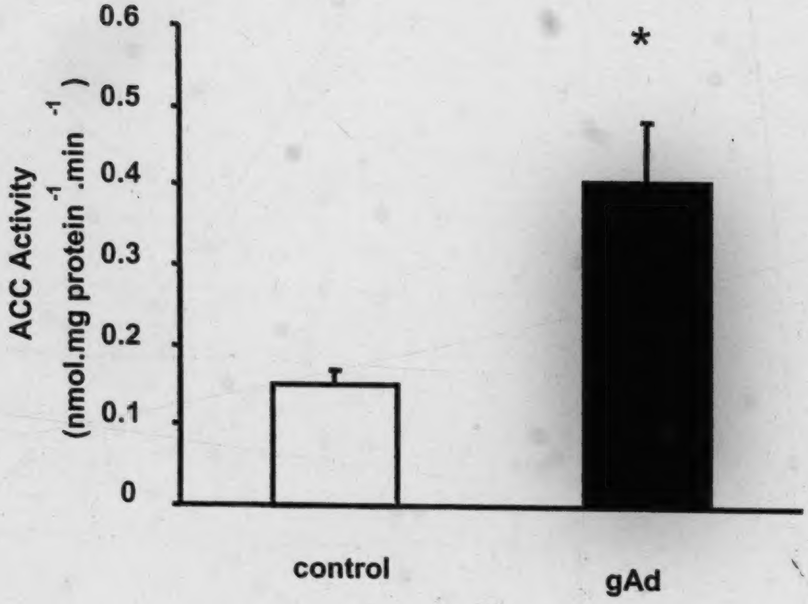


Figure 6.4. Effect of gAd on ACC activity in 7-day-old working hearts

ACC activity was measured in control and gAd-treated (1.5μg/ml) 7-day-old hearts as described in Chapter 2.

ACC activity was determined in whole tissue homogenates of the hearts that have been frozen at the end of 40 min perfusion.

Values are mean ± S.E. of 6 in control and 9 in gAd-treated hearts.

* significantly different from control hearts.

Figure 6.5. The effect of gAd on the recovery of cardiac work in 7-day-old rabbit hearts reperfused following ischemia

Seven-day-old rabbit hearts were perfused in the presence and absence of gAd (1.5 μg/ml) for an initial 30 min. A 40 min global ischemia was introduced for 40 min, following which hearts were reperfused for another 40 min.

Cardiac work was calculated as the product of peak systolic pressure and cardiac output.

A. Cardiac work in aerobic perfusion and reperfusion periods.

B. Percentage of recovery of cardiac work. The values were calculated based on the cardiac work of the hearts in the aerobic perfusion period.

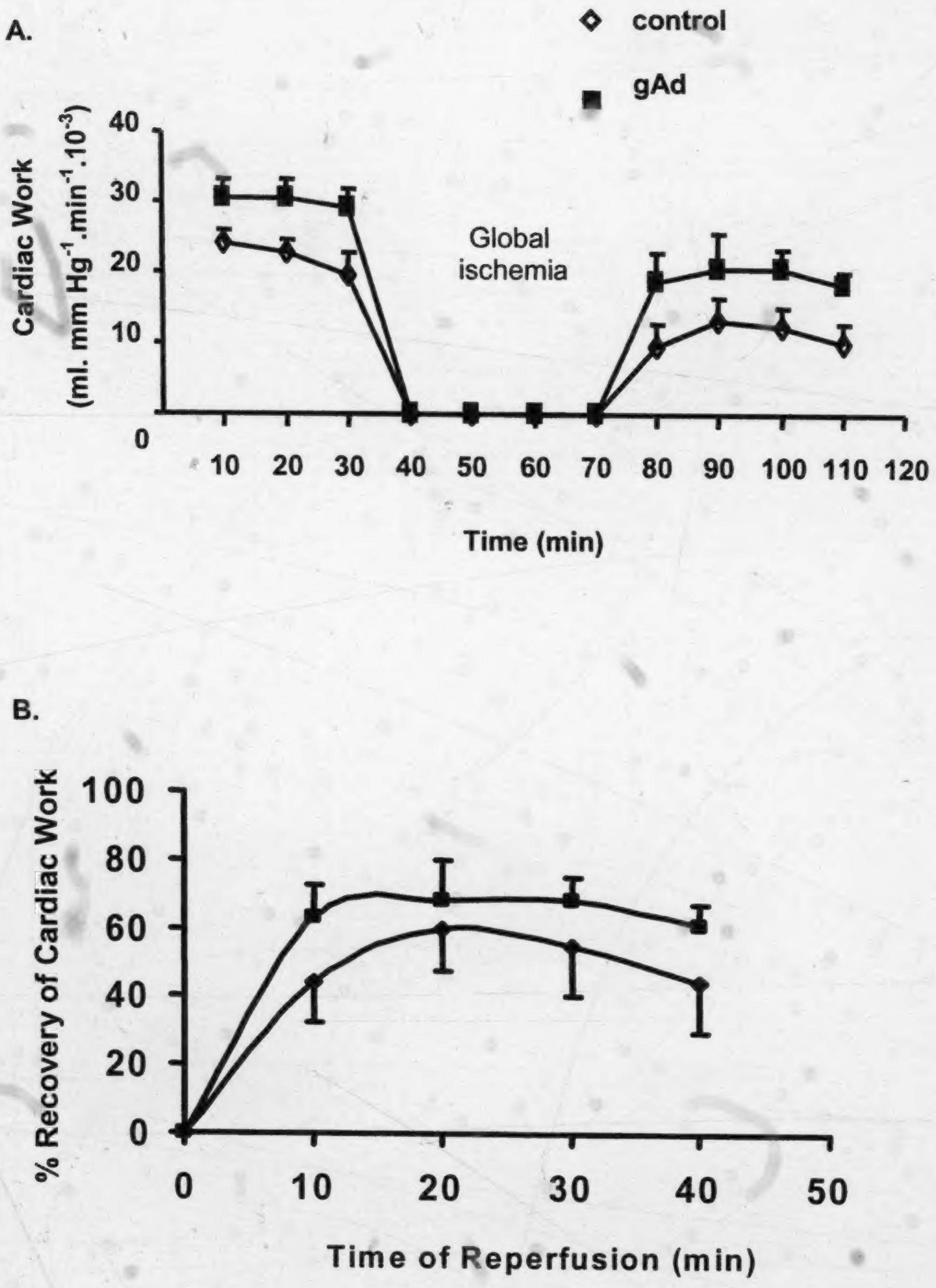

A.
control
gAd
Cardiac Work
(ml. mm Hg^{-1}.min^{-1}.10^{-3})
40
30
20
10
0
Global
ischemia
10
20
30
40
50
60
70
80
90
100
110
120
Time (min)
B.
% Recovery of Cardiac Work
100
80
60
40
20
0
0
10
20
30
40
50
Time of Reperfusion (min)

Table 6.2. Metabolic parameters in the setting of ischemia-reperfusion

	Aerobic Perfusion		Reperfusion	
	Control	gAd	Control	gAd
Palmitate oxidation *(nmol.g dry wt^{-1}.min^{-1})*	409 ± 73	505 ± 35	283 ± 82	271 ± 61
Glucose oxidation *(nmol.g dry wt^{-1}.min^{-1})*	401 ± 54	617 ± 103	458 ± 72	517 ± 91

Seven-day-old rabbit hearts were perfused in the presence and absence of gAd for an initial 30 min. A 40 min global ischemia was introduced. Thereafter, the hearts were perfused for another 40 min.

Glucose oxidation and palmitate oxidation rates were measured as described in detail in Chapter 2.

Values are mean ± S.E. of 8 control and 11 gAd-treated hearts.

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Vionnet, N., Hani, El-H., Dupont, S., Gallina, S., Francke, S., Dotte, S., De

Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, Kadowaki T. *Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase.* Nat. Med. 8: 1288-1295, **2002**.

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Chapter 7

General Discussion, Future Directions, and Limitations of This Study

7.1. Newborn Heart as a Model

All aspects of the fetal-to-newborn transition fascinate me. At first, it seems like a logical series of events that enable a smooth adaptation to extrauterine life. The placenta is not permeable to fatty acids in many species but does produce large amounts of lactate (Battagglia and Meschia 1978, Girard et al 1992, Girard et al 1985). Glucose and lactate are the major substrates the heart relies on for energy production in fetal life (reviewed in Lopaschuk et al 1992, Makinde et al 1998). Considering that only 2 mol of ATP is produced per 1 mol of glucose metabolized through the glycolytic pathway, whereas 129 moles of ATP is produced per 1 mol palmitate oxidized in the mitochondria, the fetal heart needs, and therefore produces, relatively less energy. This is probably the case, since in the uterus the fetal heart does not have to pump against a systemic resistance.

The entire organism as well as the heart has to withstand a brief period of starvation before being fed with the mother's milk. Probably, available endogenous stores of triglycerides and glycogen are mobilized immediately to supply for the increasing demand of the newborn heart. This is partially achieved by adaptations in the hormones that regulate metabolic processes. Hence, plasma glucagon increases and plasma insulin decreases in hours following birth (Bassett and Alexander 1971, Blazquez et al 1974). The high fatty acid content in the mother's milk is an excellent source for providing the increasing demand of the organism. Even the stress of birth (cold exposure, cord cutting, temperature change) is involved in the adaptation of the metabolism to the extaruterine life. In fact, the hormonal changes are suggested to be a result of the stress-related activation of the sympathetic nervous system (Girard et al 1974, Girard and Sperling 1983).

Therefore, the newborn period is unique and provides an interesting model to examine not only the metabolic changes, but also the effects of hormonal changes on the heart.

7.1.1. Is the Increase in Fatty Acid Oxidation Related To Intracellular Carnitine or the Change in CPT I Isoform Expression?

The first question I wanted to answer was based on a study by Brown et al (1995). Carnitine is primarily synthesized by the liver and to a lesser degree by the kidneys from the amino acid precursors lysine and methionine (Bremer 1983). Infants are also supplemented with carnitine from the mother during the suckling period. However, as shown by Davis (1989) the rat fetus contributes approximately 50% to its own carnitine stores by endogenous synthesis. In the study by Brown et al (1995), the authors suggested that the plasma levels of carnitine are rate-limiting in the maturation of fatty acid oxidation in the immediate newborn period.

In this study, I not only show that the total carnitine is not different between 1-day and 14-days of age, but also that the intracellular concentrations of free carnitine are above the Km of CPT I (Chapter 3). Brown et al (1995) also suggested that L-CPT I is predominantly expressed in the fetal and newborn and M-CPT I is expressed in the adult heart. However, this conclusion was not based on mRNA expression or protein expression using specific antibodies for either isoform of CPT I. Here we measured mRNA expression for both isoforms at different stages in the immediate newborn period. We did observe some changes in the mRNA expressions of L-CPT I and M-CPT I. However, the expression pattern did not parallel the increase in the newborn period. In other words, M-CPT I did not increase until 10-days of age whereas fatty acid oxidation already matures by 7-days in the newborn period.

We were not able to examine if the mRNA expression was translated into protein expression since specific antibodies for the two isoforms are not commercially available. Dr. Gebre Woldegiorgis kindly sent us antibodies for L-CPT I and M-CPT I. However the antibodies did not cross-react with the proteins from rabbit hearts.

In the study of Brown et al (1995), a radioactive-labeled form of an irreversible inhibitor of both CPT I isoforms, [3H]etomoxir, was used to examine the quantity of the enzyme (Weis et al 1994a, Weis et al 1994b, Brown et al 1995). There are two problems related to the use of etomoxir: 1) the binding characteristics of etomoxir to the two isoforms have never been characterized precisely, which is a difficult task to do due to the lack of specific antibodies for the proteins, and 2) even if it is assumed that etomoxir binds equally to both isoforms, incubation of etomoxir with a crude mitochondrial fraction prepared from the heart tissue may be misleading since etomoxir will also bind L-CPT I and M-CPT I from peroxisomal or microsomal origin.

7.1.2. Does Malonyl CoA Control Fatty Acid Oxidation when the Newborn Heart is Supplied with Increased Fatty Acids?

The key role of malonyl CoA in regulating fatty acid oxidation is becoming increasingly recognized. In the studies that I report in Chapter 4, I challenged the isolated working 7-day-old rabbit heart with high concentrations of fatty acid. It is known that the increase in fatty acid supply to the heart results in increased fatty acid oxidation rates in the adult heart. The reasons that led me to examine the situation in the newborn heart are some observations that have been reported earlier by our laboratory. Although malonyl CoA levels are lower compared to 1-day-old hearts and fatty acid oxidation is already mature by 7-days of age, increasing fatty acid supply from 0.4 mM (physiological) to 1.2 mM (pathological) resulted in a further decrease in tissue malonyl CoA levels.

An important question that remains to be answered in future studies (not only in the newborn but also in the adult heart) is whether MCD has a higher affinity for malonyl CoA in situations were malonyl CoA levels decrease. Cytosolic levels of malonyl CoA may decrease with a higher activity of MCD. If phosphorylation/dephosphorylation is involved in the acute regulation of MCD activity it may be possible to detect the changes in Vmax simply by using the maximal concentrations of the substrates in the enzyme activity assays. However, if the affinity of MCD increases for malonyl CoA, enzyme kinetics must be performed using increasing levels of malonyl CoA and determining the Km for MCD. Increase of enzyme sensitivity is not an unusual mechanism in the regulation of enzyme activity in metabolism. For instance, under conditions of increased fatty acid oxidation such as diabetes and starvation, L-CPT I of the liver becomes more active and less sensitive to the inhibition by malonyl CoA. The decrease in the sensitivity is accompanied by a decrease in tissue of malonyl CoA that allows maximal fatty acid oxidation (Drynan et al 1996). L-CPT I is the dominant isoform in the immediate newborn heart (Brown et al 1995, Chapter 3), and less sensitive to the inhibition by malonyl CoA compared to M-CPT I. Probably, high levels of malonyl CoA in the immediate newborn period potently inhibit even this less sensitive isoform of CPT I.

One of the major limitations of all the studies in this thesis is that we still do not have the technology to measure malonyl CoA levels in subcellular fractions in the heart. The malonyl CoA levels that I report in this thesis are above the inhibitory concentration for CPT I. Therefore, one can assume that CPT I in the heart is inhibited at all times by malonyl CoA. However, as suggested several times in the literature, all of the intracellular malonyl CoA is probably not in close contact with CPT I.

7.1.4. Is Adiponectin/gAd Involved in the Maturation of Fatty Acid Oxidation in the Newborn Heart?

A big challenge that I faced was to measure the plasma levels of adiponectin order to choose the concentration of adiponectin and gAd for the perfusion studies. I wanted to examine the effects of both adiponectin and gAd in the newborn hearts. I used an antibody that is raised against the gAd part of the protein. Therefore in theory, I should have been able to detect the gAd signal in the western blot analysis of the plasma samples. The signal that I observed on the immunoblots was consistently very weak, and did not allow me to perform reliable densitometric analysis. I did, however, measure the plasma concentration of the full-length adiponectin in 1-day, 7-day, and 6-week-old rabbit hearts using a quantitative western blotting technique. Plasma levels of adiponectin were very high compared to the levels of other hormones as reported by this study, as well as other studies in the field. Adiponectin exists as several molecular weight forms in the plasma (Scherer et al 1995). This raises questions as to the precise composition in the plasma, and more importantly, what is the active form of this protein. Recently, Tsao et al reported that the largest species (apparent molecular mass, 410 kDa) produced by E. Coli was an adiponectin hexamer (Tsao et al 2002). However, adiponectin also forms high molecular weight species of apparent molecular weight ~629 kDa in the plasma of both mice and humans (Tsao et al 2002, Pajvani et al 2003). In the initial perfusions, I included insulin (10 μU/ml) to the perfusate. Addition of full-length adiponectin at a concentration that simulates its plasma levels by 7-days did not affect fatty acid oxidation rates when insulin was present. gAd was also without effect under these conditions. The levels of plasma adiponectin that we report in this study are based on the trimeric structure that has a molecular weight of 90 kDa (3x 30 kDa). When selecting the concentration for treatment, we based our calculations on the trimeric form of adiponectin. We used a bacterial expression system (E. Coli) to prepare the full-length adiponectin in our laboratory. The protein mixture prepared by this system was reported to contain the trimeric and hexameric forms of adiponectin, but not the high molecular weight (HMW) form (Tsao et al 2002, Berg et al 2002). Therefore, the lack of effect with adiponectin treatment in our perfusions may be the result of the absence of the HMW form of adiponectin in our preparation. However, a recent study by Yamauchi et al (2003) showed that there are two receptors, AdipoR1 and AdipoR2, and AdipoR1 is predominantly expressed in skeletal muscle. They also showed that AdopoR1 is the high-affinity receptor for gAd. Therefore, the lack of effect of full-length adiponectin may be associated with the lack of presence of AdipoR1 receptor in cardiac tissue.

The possible interference of adiponectin with insulin is an important point. Current information on adiponectin suggests that adiponectin actually increases the sensitivity of the liver to the inhibitory effects of insulin on glucose production (Berg et al 2001). Therefore, adiponectin is suggested to act as an "insulin sensitivity enhancer". Is it possible that the presence of insulin antagonizes the effects of adiponectin whereas adiponectin actually increases insulin sensitivity? Although highly speculative, the two hormones probably have opposite effects on a common intracellular event. I believe that both gAd and insulin affect the tissue amount of triglycerides. In fact, overexpression of gAd in ob/ob mice significantly decreased tissue triglyceride content in skeletal muscle (Yamauchi et al 2002). The triglyceride levels in the muscle and liver of wild-type mice following full-length adiponectin injection have also been reported by Berg et al (2001). In that study, adiponectin did not lower muscle or triglyceride content. The reason for this discrepancy between the two studies may be the use of gAd in the study by Yamauchi et al (2002), and full-length adiponectin in the study by Berg et al (2001). The presence of insulin might have antagonized the effect of adiponectin on cardiac triglyceride content.

Yamauchi et al (2002) demonstrated that gAd and full-length adiponectin increased fatty acid oxidation, glucose uptake and lactate production via stimulating AMPK in C2C12 myocytes. In agreement with these findings, Tomas et al (2002) recently reported that gAd stimulates fatty acid oxidation in skeletal muscle by activating AMPK and inactivating ACC. We therefore wanted to examine what effect adiponectin and gAd has on metabolic parameters. gAd did not affect palmitate oxidation in 7-day-old hearts. Surprisingly, we observed and increase in glucose oxidation, and this increase in glucose oxidation was associated with an increase in cardiac work. Stimulation of glucose oxidation in the adult heart (Broderick et al 1993, McVeigh and Lopaschuk 1990), and an overall increase in oxidative metabolism in the immature heart (Itoi and Lopaschuk 1996) have been shown to improve the recovery of the heart after ischemia. We, therefore, examined the effect of gAd in the setting of ischemia-reperfusion and observed and improved recovery in gAd-treated hearts compared to the control hearts. Unfortunately, our experiments did not allow us to determine the exact cause-effect relationship between the stimulation of glucose oxidation and increased demand of the cardiac muscle.

7.2. Future Directions

Many questions still remain to be answered in future studies:

1. Does the sensitivity of MCD for malonyl CoA increase in situations in which malonyl CoA levels decrease?
2. How does the transport of fatty acids occur despite such high levels of malonyl CoA?
3. Does adiponectin have a positive inotropic effect?
4. Does adiponectin have different receptors on different tissues such as the heart, the liver and the skeletal muscle?

   This point has been covered by the recent study by Yamauchi et al (2003) while we were preparing this thesis.
5. What is the signal that cleaves adiponectin to produce gAd?
6. What is the signal that degrades the HMW complex to lower molecular weight forms?

7.3. Limitations of this Study

A major limitation of this study was that we were not able to compare the metabolic and functional parameters between 1-day and 7-day old hearts. The reason for that was the presence of *foramen ovale* in 1-day-old hearts. Instead, we investigated the effects of gAd in two age groups.

We did not measure the amounts of acetyl CoA and malonyl CoA in the subcellular compartments. Instead, we measured and reported the total amounts in whole tissue homogenates.

The recombinant adiponectin that we used in our perfusions does not include all the forms that appear in the organism. The lack of the effect of full-length adiponectin may be related to the absence of higher molecular weight structures in our preparation.

We were not able to measure the amount of gAd in the plasma. The concentration of gAd that we used was based on literature values and may not simulate its physiological concentration.

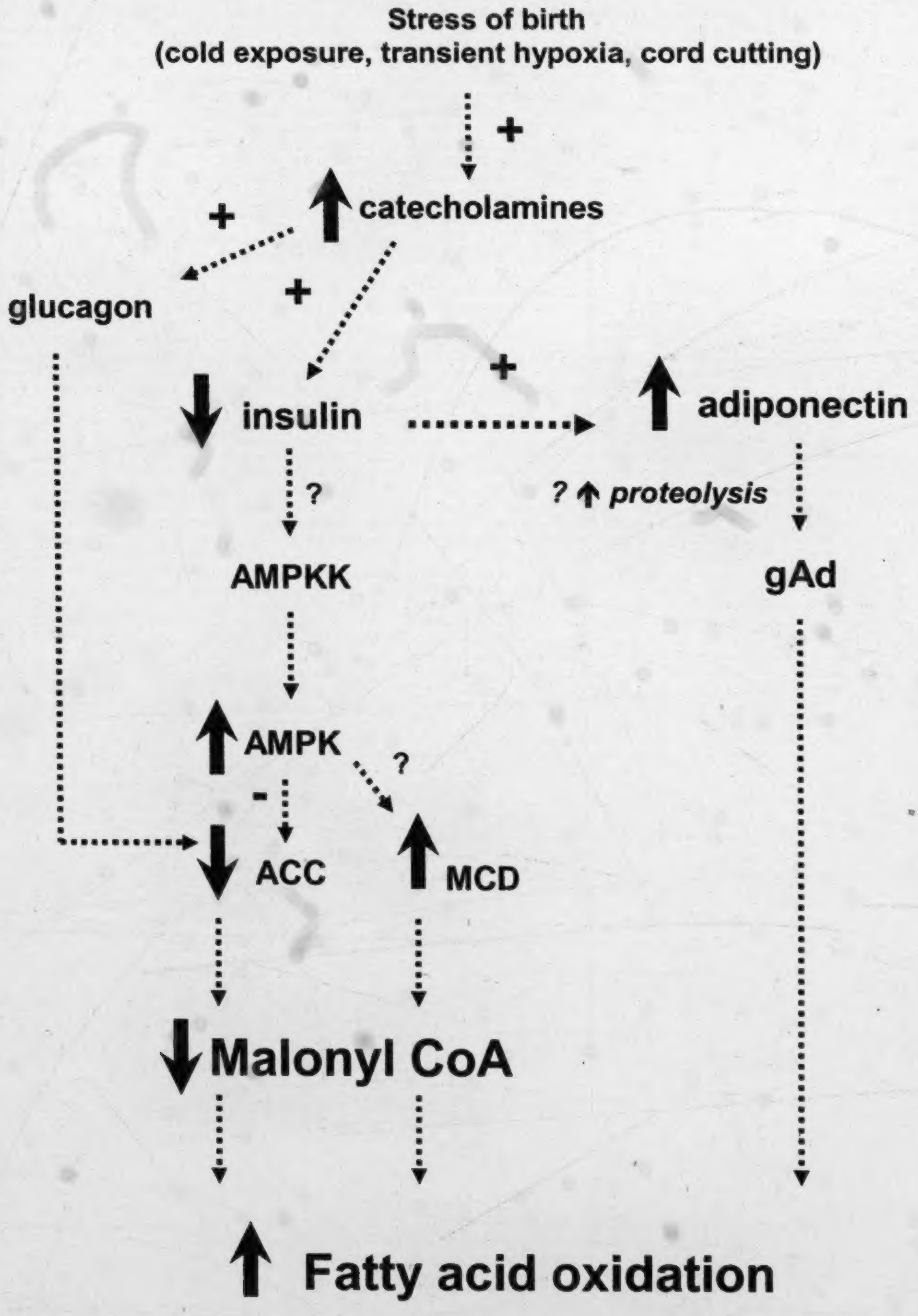
7.4. Summary - What is responsible for the maturation of fatty acid oxidation in the newborn heart?

1. Myocardial carnitine levels do not change in the newborn period. The tissue amount is sufficient to allow fatty acid oxidation to occur even in 1-day-old hearts.
2. The change in isoform expression pattern of CPT I does not parallel the increase in fatty acid oxidation.
3. The IC_{50} of CPT I for malonyl CoA is not different between 1-day and 10-day old hearts.
4. Cardiac MCD activity increases in the newborn period.
5. Malonyl CoA regulates fatty acid oxidation rates even when the newborn heart is exposed to high levels of fatty acids.
6. Plasma levels of adiponectin increase in the newborn period.
7. gAd stimulates fatty acid oxidation in 1-day-old hearts.
8. The presence of insulin antagonizes the effects of gAd on fatty acid oxidation.
9. The increase in fatty acid oxidation is AMPK-independent.
10. Cardiac AMPK activity increases between 1-day and 7-day.
11. AMPK activity is lower when insulin is present in the perfusate.

Based on this information the scheme in Figure 7.1. is proposed.

Figure 7.1. Proposed pathway by which fatty acid oxidation increases in the newborn heart

Plasma glucagon increases and plasma insulin decreases as a result of sympathetic activation and increase in catecholamine release. An increase in both AMPK expression and activity results in a decrease in ACC activity. The increase in glucagon contributes to the inhibition of ACC activity via (Protein kinase A) PKA. MCD expression and activity increases. Following birth, a decrease in ACC activity and increase in MCD activity results in a dramatic drop in malonyl CoA. The decrease in insulin triggers adiponectin/gAd release from the adipocytes. The increase in adiponectin stimulates fatty acid oxidation.


Stress of birth
(cold exposure, transient hypoxia, cord cutting)
+
catecholamines
+
glucagon
+
+
insulin
adiponectin
?
? proteolysis
AMPKK
gAd
AMPK
?
-
ACC
MCD
Malonyl CoA
Fatty acid oxidation

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